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UNPUBLISHED PRELIMINARY DATA

THE GENERAL AND COMPARATIVE BIOLOGY OF TERRESTRIAL ORGANISMS UNDER EXPERIMENTAL STRESS CONDITIONS *

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I. INTRODUCTION

Biologists have worked according to engineering specifications and standards in the study of simulated extra-terrestrial environments, yet the biological information generated has not constituted a fair return on the investment of time, labor and materials in the electronics and "hardware" required.

We cannot hope to duplicate the "environment" of Mars in the laboratory, because any planet possesses a virtual infinity of environments consisting of many elements in a state of continuous change. Furthermore, the limitations inherent in methods of remote measurement (spectroscopy, radiometry, etc.) make it impossible to determine with certainty even an average set of extra-terrestrial conditions.

We can, however, approximate some extra-terrestrial conditions remarkably well, others passingly well, and some poorly or not at all. The individual factors, properly evaluated, can be combined into a near simulation of a specific environment. But only by the study of various factors individually can the nature of their interplay be understood.

Our approach rests heavily upon the concept that the total of earthly environments is part of a greater bio-ecological continuum within the Cosmos, and that virtually any rational and systematic alteration in the factors which comprise a terrestrial condition must approximate a part of some other planetary eco-system, known or unknown. In other words, the raison d'etre for environmental simulation as an experimental tool is the evaluation of the capabilities of life forms familiar to us in order that we may better assess the directions in which they might depart if the environment and selection pressures changed in a particular manner.

Specifically, we may select particular factorial combinations which can be designated "simulated high altitude", "simulated Martian equatorial summer", etc. Then, having in mind the factors singly and in lower orders of combination, we can move in simile from Earth to 60,000 feet, toward Mars, or in any other reasonable

direction with some confidence that the biological performance - survival, acclimatization, adaptation - will give us meaningful information toward a picture of organic evolution on a cosmic scale.

If this philosophy and methodology are adhered to, we believe that a host of new morphological and biochemical phenomena, as well as practical techniques such as extra-terrestrial farming, will surely make their appearance as a matter of course.

The original research plan submitted to the National Aeronautics and Space Administration proposed a study of seed plants under atmosphere, temperature, and water stress, the three factors to be examined singly and in combinations. Other environmental factors, such as substratum and radiation, and modifications in physiological and biochemical processes were also to be considered. During the period of negotiations in 1962 and 1963 prior to award of the contract, the above plan together with other work of a comparative nature involving animals as well as plants was carried out. Accordingly, our program is far broader in scope than called for in the original proposal, and some aspects of the original plan were well advanced prior to the actual starting date of Contract NASw-767, July 1963.

Many of the results reported below are to be published in "Current Aspects of Exobiology", a book edited by M. Briggs and G. Mamikunian under the chapter heading, "The Performance and Capabilities of Earth Organisms in Simulated Extra-terrestrial Environments". Publication will be no earlier than late 1963.

Those data dealing with the primary factorial study of seed germination revised and brought up to date during the contract period are presented in the body of the text. Other investigations relevant to seed plants follow factorial studies and preliminary multiple factor tests.

Other plant and animal data acquired prior to the first quarter are to be found in the appendices to this report.

II. EXPERIMENTAL PROCEDURES

A. Selection of Reference Systems - Emphasis has been placed upon variations in partial pressure of oxygen and of total pressures, because it is reasonable to suppose that the universe offers many anaerobic conditions, as well as aerobic environments covering a range of partial and total pressures. Indeed, the microenvironments on this planet cover an appreciable range of oxygen pressures. Convenient reference points include the biotic zone of the Himalayas (elevation: 20,000 feet, $P_{total} \sim 380$ mm., $P_{O_2} = 76$ mm.); air pressures equivalent to 50,000-60,000 ft.; and completely anaerobic conditions.

Pure O_2 at 1 atm. is assumed to be rare in nature but is useful to study the phenomenon of oxygen poisoning, a possible hazard in artificial environments. Furthermore, oxygen in the normal atmosphere will rapidly kill plants grown in a low oxygen or oxygen-free atmosphere.

Additional factors are temperature cycles of several kinds, including +20 to 25°C day temperature (8 hrs.), -20 to -30°C night temperature (16 hrs.) which is adopted as a summer equatorial temperature cycle for Mars. Cycles involving other times and temperatures are included as well as single high and low temperature shock treatments.

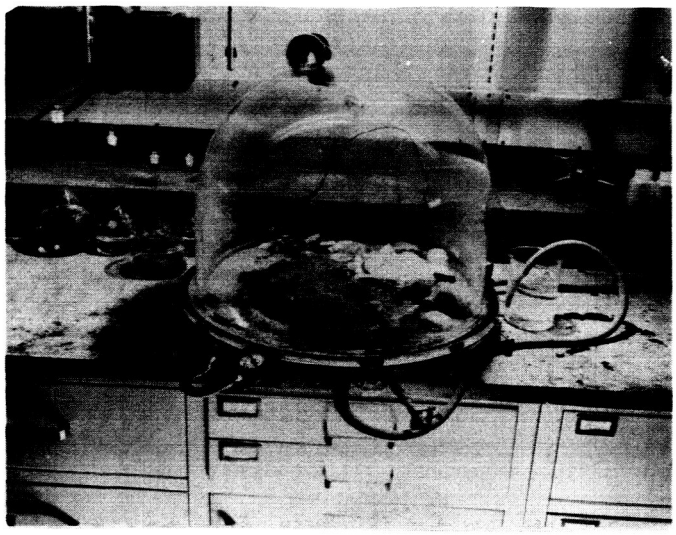
Water supply is one of the most controversial factors in a simulated Martian environment, and a variety of experimental variations have been devised. Values for condensable atmospheric water in the range of 0.01-0.05 gm/cm^2 have been adopted. Ar alternative procedure is the application of 1 cm of water ice to the substratum at 2-4 week intervals.

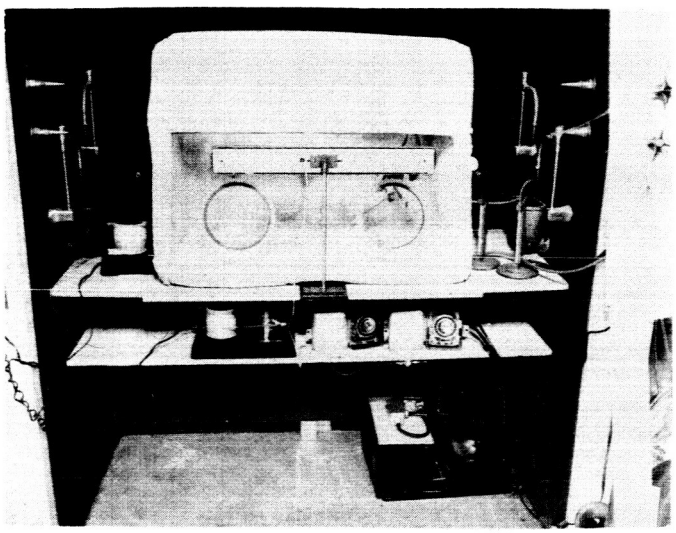
Factors such as centrifugal simulation of gravity, radiation, and substratum have so far received only preliminary study.

Other factorial combinations include the anaerobic desert, which is used in a chamber operating at P = 1 atm., 100% N_2 or 98% N_2 + 2% CO_2. Dew Point -60°C; temperature ~ 25°C; and the diurnal freezing cycle under aerobic conditions.

The most important reference system to be considered here is the "Nearly-Simulated Martian Equatorial Summer". This environment is approached factorially as a tri-partite biological screen for competence of seed germination at suitable temperature, atmospheric and water conditions; and is then followed by the combination of factors. This approach will be considered in detail below.

A number of facilities have been used for establishing single- and multi-factorial simulated conditions (Fig. 1). The large Plexiglas dome used for turtles and lower plants is equipped for gassing, watering, draining and feeding. The small Plexiglas dome for seed and insect work has a refrigerator coil for liquid nitrogen and an electric vibrator for dislodging frost from the coil to simulate snow (frost) fall. Both domes can be operated at reduced pressures. The large, foam-insulated box may be operated anaerobically and programmed for specific warm-light and cold-dark cycles. The anaerobic desert is a chamber which passes 3-4 liters/min. of N_2 or N_2 + CO_2 at Dew Point -60°C.





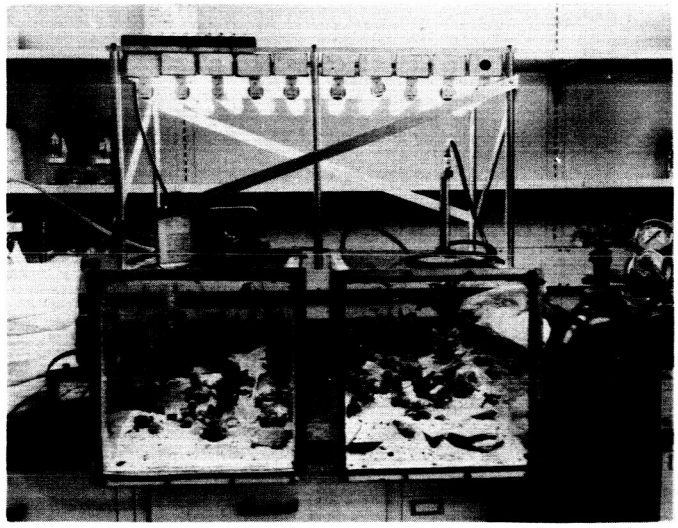


Fig. 1 Environment Simulating Equipment
Upper Left: Large Plexiglas Chamber used for Turtle Colony
Upper Right: "Snow Dome" used for controlled water supply to seeds
Lower Left: Nearly-Simulated Martian Equatorial Summer
Lower Right: Anaerobic Desert Chamber

Routing screening of atmospheric effects on seed germination, insect behavior, and other processes have been studied in 4 or 16 liter anaerobic jars (Fig. 2), which can be placed in controlled temperature and light conditions.

B. Organisms - Nearly 300 species and varieties of seed plants, 24 species of lower plants and protista, and over 20 species of animals have been studied in various ways in this laboratory (Table 1). In a few instances, selections have been made on a specific ecological basis. For example:

1. The peanut was expected to germinate with little or no O_2, in recognition of the subterranean development of the peanut seed.
2. Some of the grasses and cereals are native to cold semi-arid regions of relatively high altitude, hence were expected to withstand appreciable cold at reduced O_2 levels.

The protista and lower plants on the other hand were viewed as more or less direct descendents of forms which may have lived when the atmosphere had far less O_2 than at present. Seed selection was made principally on a random basis, whereas the turtle was chosen purely by intuition.

The most striking biological performance in many respects was shown by organisms which had not been introduced intentionally in the "Mars Equatorial Summer" and "Anaerobic Desert" conditions. Fungi, presumably introduced as spores, grew well during simulator operation. The species isolated in pure culture and identified will be discussed below.

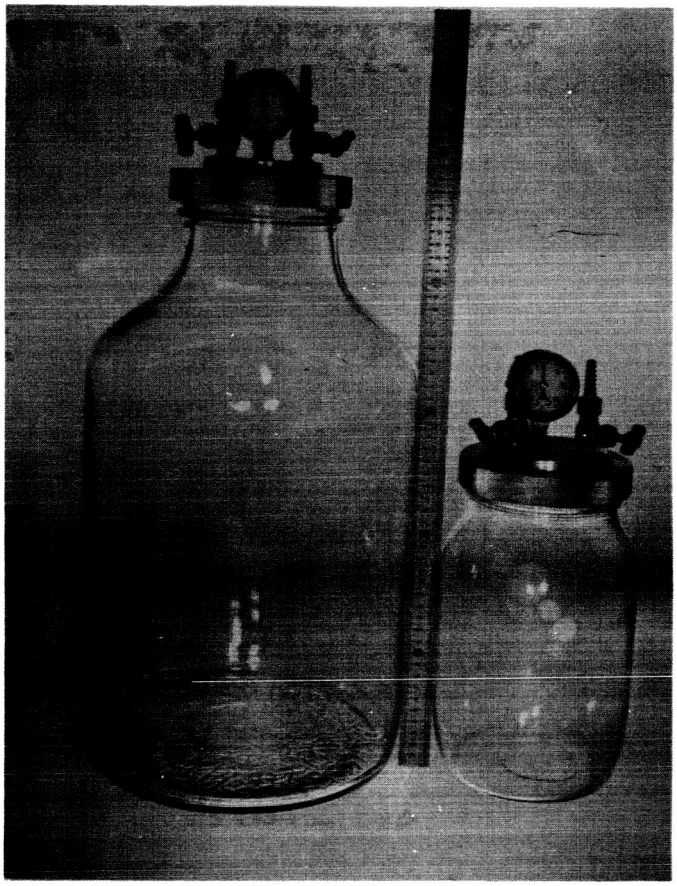


Fig. 2. Anaerobic Jars used in Routine Operations

Table 1

Organisms Studied

Group		Examples	Extent of Testing	Environments Used	Basis for Selection
Seed Plants		Legumes Cereals Composites Mustards	Nearly 300 species and cultivated varieties	General, Low O_2, P_{O_2} 760 mm	Most random, some ecological
Cryptogamic Plants	Autotrophic	Ferns Mosses Hornworts and Liverworts	6 species	Low pressure, Low O_2, P_{O_2} 760 mm Low water	Paleobotanical
	Heterotrophic	Fungi	About 12 species, not all identified	Contaminants in anaerobic desert and Mars factorial	By their presence in simulators
Protista		Mastigophora Ciliata	6 species	Low pressure	Paleontological
Metazoa Invertebrate		Coelenterata Platyhelminthes Nemathelminthes Mollusca Insecta Crustacea	20 species	Low pressure, Low O_2, P_{O_2} 760 mm Low temperature, salinity and desiccation	Part random, Part ecological
Vertebrate		HeLa Cell Amphibia (tadpoles) Reptilia (turtles)	One species of each	Low O_2, P_{O_2} 760 mm freezing Simulated high altitude	Principally biological intuition

C. Standards of Response and Performance - The simplest order of response under environmental stress is death. Of course, up to a point test organisms in process of failing may be rescued from some situations. The standards of performance can be summarized as follows:

Stage	Designation	Condition	
		Plant	Animal
1st	Suitable Environment - no challenge or stress	Growth and reproduction	Growth, Reproduction, Locomotion (if normal)
2nd	Retardation, sub-optimal conditions, moderate stress	Growth, little or no reproduction	As with plants. Locomotion impeded.
3rd	Survival (Maintenance)	No growth, inactivity or dormancy	As with plants
4th	Failure	Progressively irreversible decline in function and structure	
5th	Death	Irreversible Loss of Function	

Two mechanisms for circumventing failure, acclimatization and dormancy, would be expected to occur between stages 3 and 4, that is before the process of decline. The failing organism may recover by direct resuscitation, or regeneration of severely damaged structures may be required. The ways in which organisms exhibit failure or other responses will be detailed later.

Genetic heterogeneity within a population will have a bearing on stress response, and it appears that new areas of phenotypic expression result from experimental environments which create hitherto non-existent (or rare) types of selection pressure.

III. THE FACTORIAL APPROACH TO SPECIFIC SIMULATION

In the preceding sections, a variety of conditions and responses have been described and discussed in more or less detail, and random references have been made to Martian conditions. In order to evaluate the potentialities of biological systems under simulated or nearly simulated Martian Equatorial summer conditions, a factorial screen was set up for three major determinants of the Martian environment complex. Seed germination was selected as a means for bio-evaluation of atmosphere, temperature, and water availability.

A. Screening Seed Populations for Primary Capabilities

Screen 1 was set up to select for anaerobic species, varieties, or individuals in seed populations. The atmosphere was argon which may contain traces of O_2. No CO_2 was introduced, but was generated by the seeds themselves, raising the chamber level to about 2-3% by the end of the test period.

Screen 2 was devised to test for "cryobic" forms by incubation of seeds on the diurnal patterns: 4°C, 16 hrs./22°C, 8 hrs.; -30°C, 8 hrs./+22°C, 16 hrs.; and -30°C, 16 hrs./+22°C, 8 hrs.

Screen 3 was devised to select for xerobic forms; that is, seeds capable of germinating against a water deficit. Two media were used for incubation, namely 10% agar and 1 M mannitol. Osmotic methods for creating a water deficit may or may not be physiologically distinguishable from literal xeric conditions. Additional techniques must certainly be used as well. We believe that work against a concentration gradient and extraction of water from a hydrophilic colloid are means for studying the water-accumulating efficiencies of various species.

Screen 1 shows three patterns of response. Some populations consist of obligate aerobes; others contain a minor percentage of facultative anaerobes (5b, 17, 40b, for example) in a major population of strict aerobes; and some populations consist of significant proportions — or almost entirely — of facultative anaerobes (7a, 62, 144, for example). Extreme varietal differences are apparent in some cases — in species 7 (Celosia argentea) and species 80 (Cucumis sativus), for example.

Of 249 species and varieties given, 15% are weakly anaerobic (< 15% germination) and 9% are strongly anaerobic. In all, screen 1 required the use of a total of about 70,000 seeds.

Screen 2 presents a selection of 41 species from screen 1, half of which have anaerobes in the population. Those capable of any germination at all on the 16 hr. -30°C cycle are unquestionably low-temperature competent. There are five such species, and all of them are anaerobic to some degree. Eighteen species germinate to some extent on the short sub-freezing cycle, and they are equally divided between aerobic and anaerobic classifications. However, to be more selective, only those showing 90% germination or more on the 8 hr. at -30°C cycle were ranked with the 5 from the 16 hr. -30° condition. Of this 4, 3 are anaerobes. Hence, 8 out of 9 of the forms showing good low temperature performance represent populations containing anaerobic members.

Screen 3 shows that only 6 species can germinate against osmotic stress: the great difference between mannitol and agar suggests that the latter be discarded as a reference standard. Arbitrarily, the two species 68b and 7c which germinate over 90% on agar are included with those succeeding in mannitol. Of these 8 species, half performed well on screen 2, half did not; 5 out of 8 were from populations with anaerobic abilities. There appears to be a positive relationship between anaerobic and cryobic abilities, but only a random relation between these factors and osmotic stress.

If the 28 species receiving triple screening are evaluated for overall performance by adding their "+" responses, 3 receive "0", that is, show no stress capabilities; 17 have only single-factor competence; 4 have two-factor capabilities. The final group, representing triple-factor capability consists of 7c, Celosia argentea "Forest Fire"; 70, Brassica pekinensis; 77d, Raphanus sativus "Scarlet Turnip White Tip"; and 101, Secale cereale "Winter". Of these, Winter rye is the most outstanding and cockscomb is second. The other two, both members of the mustard family (Cruciferae), are relatively poor anaerobes.

FAMILIAL LISTING OF GENERA, SPECIES AND VARIETIES OF SEED SCREENED FOR ANAEROBIC CAPABILITIES

Family	Genus, Species, Variety	Germination Percentage* (After 5 Days at 25°C)
Acanthaceae	1. Thunbergia alata	0
Aceraceae	2. Acer platanoides	0
	3. Acer saccharinum	0
Aizoaceae	4. Cryophytum crystallinum	0
Amaranthaceae	5. Amaranthus caudatus	
	a. Globe Buddy	0
	b. Molten fire	5
	6. Amaranthus tricolor	5
	7. Celosia argentea	
	a. Empress	92
	b. Fire Feather	48
	c. Forest Fire	50
	d. Giant Plume Golden Feather	9
	e. Golden Feather	36
	f. Toreador	33
Amaryllidaceae	8. Allium Cepa	
	a. Southport yellow Globe	2
	b. Evergreen Bunching	3
	c. White Portugal	3
	9. Allium Porrum	
	a. American Flag	2
Apocynaceae	10. Vinca minor	0
Balsaminaceae	11. Impatiens Balsamina	0
Begoniaceae	12. Begonia Pieta	0
	13. Begonia semperflorens	0
Boraginaceae	14. Anchusa azurea	0
	15. Borago officinalis	0
	16. Cynoglossum amabile	0
	17. Heliotropium arboresens	1
	18. Myosotis scorpioides	0
Cactaceae	19. Cephalocereus sinilis	0
	20. Mammilaria, sp.	0
	21. Opuntia sp.	0
Campanulacae	22. Campanula medium	0
	23. Campanula rotundifolia	0
Capparidaceae	24. Cleome spinosa	0
Caryophyllaceae	25. Cerastium tomentosum	0
	26. Dianthus barbatus	0

*Based upon triplicates totalling at least 300 seeds

	27. Dianthus Caryophyllus	
	a. Chabauds Giants	15
	b. Sparkly Bright Scarlet	6
	28. Gypsophila paniculata	
	29. Lychnis chalcedonica	1
	30. Lychnis viscaria	0
Chenopodiaceae	31. Beta vulgaris	
	a. Detroit Dark Red	0
	b. Ruby Queen	0
	c. Wonder	0
	d. var. cicla	6
	32. Kochia scoparia	0
	33. Spinaca oleracea	
	a. Bloomsdale Savoy	0
Compositae	34. Achillea Ptarmica	0
	35. Artemisia dracunculus	0
	36. Aster sp.	0
	37. Aster tanacetifolium	0
	38. Brachymome iheridifolia	0
	39. Centaurea cyanus	
	a. Bachelors Button	0
	b. Dusty miller	0.7
	c. Snowman	0
	40. Chrysanthemum sp.	0
	41. Chrysanthemum morifolium	0
	42. Chrysanthemum parthenium	0
	43. Cichorium Endivia	0
	44. Cichorium Intybus	0
	45. Coreopsis sp.	0
	46. Cosmos bipinnatus	0
	47. Cremanthodium reniforme	10
	48. Dahlia pinnata	0
	49. Dimorphothaecae sinuata	0
	50. Felicia Bergeriana	0
	51. Gaillardia sp.	0
	52. Helianthus annuus	3
	53. Helichrysum bracteatum	0.4
	54. Helipterum roseum	0
	55. Lactuca sativa	
	a. Black Seeded Simpson	0
	b. Grand Rapids	0
	c. Early Curled Simpson	0
	d. New York No. 12	0
	e. White Boston	0
	f. White Curled Simpsor	0
	56. Scorzonera hispanica	0
	57. Tagetes tenufolia	0
	58. Taraxacum officinale	0
	59. Tithonia diversifolia	0
Convolvulaceae	60. Quamoclit pennata	0
	61. Quamoclit sp.	0
	62. Convolvulus japonicus	40
Crassulaceae	63. Sedum Himalaium	0

Cruciferae	64. Aubrietta deltoidea		0
	65. Arabis procurrens		0
	66. Brassica caulorapa		0
	67. Brassica napobrassica		0
	68. Brassica nigra		
	a. Florida Broadleaf		2
	b. Giant Curled Southern		0
	69. Brassica Oleraceaea		
	a. var. acephala		4
	b. var. botrytis		0
	c. var. capitata	c_1 Jersey Queen	2
		c_2 Red Acre	4
	d. var. gemmifera	d_2^2 Jade Cross	0
		d_2^1 Catskill	0
	e. var. italica		3
	70. Brassica pekinensis		15
	71. Brassica rapa		
	a. Purple Top White Globe		0
	72. Cheiranthus cheiri		0
	73. Hesperis sp.		0
	74. Iberis sp.		0
	75. Lunceria annuus		0
	76. Matthioloa bicornis		
	a. Dwarf 10 weeks		0
	b. Trisomic 7 weeks		2
	77. Raphanus sativus		
	a. Champion		2
	b. Crimson Giant		5
	c. Round Black Spanish		3
	d. Scarlet turnip white top		1
	e. Sparkler		1
Cucurbitaceae	78. Citrullus vulgaris		0
	79. Cucumis Melo		
	a. Summer Crookneck		0
	80. Cucumis sativus		
	a. Black Diamond		90
	b. Long Green		72
	c. Straight 8		79
	d. West Indian Gherkin		62
	e. Wisconsin		29
	81. Cucurbita Pepo		0
	82. Lagenaria siceraria		
	a. Small orange		0
	b. Ornamental bottle		0
Dipsacaceae	83. Scabiosa atropurpurea		0
Ericaceae	84. Vaccinium retosum		0
Euphorbiaceae	85. Euphorbia marginata		0
	86. Euphorbia corollata		0
	87. Ricinus communis		0
Geraniaceae	88. Geranium maculatum		0
	89. Geranium sanguineum		0
Gesneriaceae	90. Saintpaulia ionantha		0
	91. Sinningia speciosa		0
Gramineae	92. Agrostis alba		0

	93. Avena sativa	
	var. Sieghafer	0
	94. Briza maxima	0
	95. Coix Lacryma - Jobi	0
	96. Festuca rubra	3
	97. Hordeum vulgare	50
	98. Oryza sativa	
	a. var. Patna	17
	100. Phleum pratense	0
	101. Secale cereale	96
	102. Triticum vulgare	0
	103. Zea Mays	
	a. Midget Sweet	75
Hydrophyllaceae	104. Nemophila Menziesii	0
	105. Phacelia tanacetifolia	0
Iridaceae	106. Iris clarkii	0
	107. Tigridia Pavonia	0
Labiatae	108. Coleus Blumei	0
	109. Lavendula officinalis	0
	110. Marrubium vulgare	0
	111. Majorana hortensis	0
	112. Mentha piperita	0
	113. Mentha spicata	0.2
	114. Molucella piperita	0
	115. Nepeta cataria	0
	116. Nepeta mussinii	0
	117. Ocimum Basilicum	0
	118. Rosmarinus officinalis	0
	119. Salvia splendens	0
	120. Satureja hortenses	2
	121. Thymus vulgaris	0
Leguminosae	122. Arachis hypogaea	
	a. Virginia Giant	50
	123. Glycine max	0
	124. Lathyrus odoratus	0
	a. Floribunda Salmon	7
	b. Shirley Temple	0
	125. Lathyrus Latifolius	0
	126. Lupinus pubescens	0
	127. Mimosa pudica	0
	128. Phaseolus multiflorus	0
	129. Phaseolus vulgaris	
	a. Black Valentine	0
	b. Red Kidney	0
	130. Pisum sativum	0
	131. Vicia faba	50
Liliaceae	132. Asparagus officinale	0
	133. Asparagus plumosa	0
	134. Fritillaria imperealis	0
	135. Kniphofia Uvaria	0
	136. Lilium gigantium	0
	137. Lilium regale	0
	138. Smilax herbacea	0

Linaceae	139. Linum usitatissimum	0
Lobeliaceae	140. Lobelia Erinus	
	a. var. compacta	0
	141. Lobelia gracilis	0
Malvaceae	142. Althaea rosea	
	a. Double Mixed	1
	b. Old Fashioned	1
	143. Hibiscus militaris	1
	144. Hibiscus esculentus	5
Menispermaceae	145. Menispermum canadense	0
Nyctaginaceae	146. Mirabilis Jalapa	1
Onagraceae	147. Clarkia elegans	0
	148. Godetia amoena	0
	149. Oenothera biennis	0
Oxalidaceae	150. Oxalis sp.	0
Papaveraceae	151. Eschscholzia californica	0
	152. Papaver nudicaule	0
	153. Papaver orientale	0
Plumbaginaceae	154. Limonium sp.	0
	155. Plumbago sp.	0
Polemoniaceae	156. Phlox divaricata	0
	157. Phlox Drummondii	0
Polygonaceae	158. Rheum mobile	0
	159. Rheum palmatum	0
Portulacaceae	160. Portulaca grandiflora	0
Primulaceae	161. Cyclamen persicum	0
	162. Primula sinensis	0
Ranunculaceae	163. Aconitum sp.	14
	164. Aquilegia canadensis	0
	165. Aquilegia sp.	0
	166. Arctotis sp.	0
	167. Delphinium Ajacis	0
	168. Delphinium cardinale	0
	169. Ranunculus sceleratus	0
	170. Thalictrum chelidonii	0
Resedaceae	171. Reseda odorata	0.7
Rosaceae	172. Fragaria virginiana	0
	173. Geum triflorum	0
	174. Rosa bracteata	0
	175. Rosa cathayensis	0
Rubiaceae	176. Coffea arabica	4
Rutaceae	177. Citrus Aurantium	0
	178. Citrus grandis	0
	179. Citrus Limon	0

Scrophulariaceae	180. Antirrhinum majus	0
	181. Digitalis purpurea	0
	182. Linaria dalmatica	
	a. var. grandiflora	0
	183. Nemesia sp.	0
	184. Veronica longifolia	0
Solanaceae	185. Capsicum frutescens	
	a. var. longum	0
	186. Nicotiana Tabacum	0
	187. Lycopersicon esculentum	0
	188. Petunia hybrida	0
	189. Physalis Alkekengi	0
	190. Salpiglossis sinuata	0
	191. Schizanthus pinnatus	0
	192. Solanum Melongena	
	a. Black Beauty	0.7
	b. Florida Highbush	0
Tropaeolaceae	193. Tropaeolum majus	
	a. dwarf	0
	b. golden gleam	7
	c. mahogany	0
	194. Tropaeolum peregrinum	0
Typhaceae	195. Typha latifolia	80
Umbelliferae	196. Anethum graveolens	0
	197. Carum Carvi	0
	198. Coriandrum sativum	0
	199. Daucus carota	0
	200. Foeniculum vulgare	2
	201. Pastinaca sativa	0
	202. Trachymene coerula	0
Verbenaceae	203. Lantana Camara	
	a. var. hybrida	0
	204. Verbena sp.	0
Violaceae	205. Viola tricolor	
	a. var. Hortensis	0

Screen 2

SELECTED SPECIES FROM SCREEN 1 TESTED FOR CRYOBIOTIC CAPABILITIES

Species No. (From Screen 1)	Screen 1 Performance	Germination Percentage After 5 Days At					
		+ 4°C 16 hrs. +22°C 8 hrs.		−30°C 8 hrs. +22°C 16 hrs.		−30°C 16 hrs. +22°C 8 hrs.	
6	+	90		0		0	
8a	+	92		100		12	
15	−	67		0		0	
26	−	90		80		0	
27a	+	92		5		5	
27b	+	90		20		3	
31d	+	0		0		0	
33	−	80		10		0	
51	+	0		0		0	
54a	−	100		0		0	
58	−	0		0		0	
62	+	40		0		0	
67	−	95		0		0	
70	+	100		0		0	
77b	+	55		33		0	
77c	+	40		73		0	
77d	+	100		100		10	
80a	+	68		84		0	
81	−	0		0		0	
94	−	38		90		0	
95	−	0		0		0	
97	+	92		0		0	
98	+	90		66		0	
101	+	98		90		20	
103	+	0		0		0	
112	−	25		5		0	
117	−	0		0		0	
122	+	16		0		0	
123	−	90		0		0	
129a	−	0		0		0	
129b	−	75		0		0	
130	−	0		0		0	
131	+	0		7		0	
139	+	0		0		0	
144	+	0		0		0	
150	−	80		80		0	
172	−	20		5		0	
183	−	25		3		0	
191	−	0		0		0	
193b	−	0		0		0	
201	−	5		39		0	

Screen 3

SELECTED SPECIES FROM SCREEN 1 TESTED FOR XEROBIOTIC CAPABILITIES

Species No. (From Screen 1)	Screen 1 Performance	Screen 2 Performance	Germination Percentage After 3 Days In 1 M Mannitol	Germination Percentage After 3 Days In 10% Agar
6	+	-	0	60
7c	+	+	0	93
8a	+	-	0	33
15	-	+	0	48
27b	+	+	0	45
31a	-	+	0	5
33	-	-	0	63
40a	-	-	0	48
51	+	-	0	36
62	+	-	0	24
67	-	-	12	18
68b	-	-	0	96
$69c_1$	+	-	0	51
$69c_2$	+	-	5	55
$69d_1$	-	-	0	25
70	+	+	18	87
77d	+	+	6	60
80a	+	+	0	21
80e	+	-	0	87
98	+	+	0	0
101	+	+	35	100
103	+	-	0	33
122	+	-	0	0
144	+	-	0	0
176	+	-	0	0
193b	+	-	0	0
196	-	-	6	9
199	-	+	0	15

Triple Screen Score

Selected Species from Screens 1, 2, and 3

Species No. (from Screen 1)	Screen 1 Performance	Screen 2 Performance	Screen 3 Performance	Overall Score
6	+	-	-	1
7c	+	+	+	3
8a	+	-	-	1
15	-	+	-	1
27b	+	+	-	2
31a	-	+	-	1
33	-	-	-	0
40a	-	-	-	0
51	+	-	-	1
62	+	-	-	1
67	-	-	+	1
68b	-	-	+	1
$69c_1$	+	-	-	1
$69c_2$	+	-	+	2
$69d_1$	-	-	-	0
70	+	+	+	3
77d	+	+	+	3
80a	+	+	-	2
80e	+	-	-	1
98	+	+	-	2
101	+	+	+	3
103	+	-	-	1
122	+	-	-	1
144	+	-	-	1
176	+	-	-	1
193b	+	-	-	1
196	-	-	+	1
199	-	+	-	1

B. The (Partially) Simulated Martian Environment - Several experiments using the full complex of environmental factors have shown that our general approach to simulation is feasible and useful if not yet optimal (Table 2a and b). Consistent with its behavior in other respects, Winter rye is the only species which has shown substantial promise in these experiments.

To date, simulation is inaccurate in the following ways:

a. The warm phase is too long.

b. The low temperature may not be low enough.

c. The atmospheric pressure is too high (760 mm) and in some cases lacked CO_2.

d. The light regime is not yet realistic in visible + ultraviolet energy distribution.

e. The substratum is too simple.

f. The water problem is not solved.

These factors are under continuous review and are subject to improvement. The method of supplying water suffers from being discontinuous. This will be rectified by distributing the ice continuously over a period of days or weeks. It should be noted, however, that the surface water condition is modified by continuous flow of dry atmosphere. Psychrometric readings have only been attempted once after addition of ice to the surface, and showed that with the existing flow-through of gas at dew point -60°C, the humidity 10 cm above the melting ice was so low that differential wet-dry bulb readings went off scale on standard tables.

The results show that in addition to its "inherent" capability, Winter rye performance can be improved by a number of chemical agents which may substitute for the oxidizing power of O_2. Quinones and nitrate may serve in this way directly. ATP itself replaces O_2 only to a small extent. Glycine may support the Stickland reaction. Combinations of these substances have a remarkably good effect, and suggest that there are two or more limiting pathways to be examined. The effect of CO_2

Table 2a - Operating Conditions and Plant Performance in Exploratory Mass Simulator Runs

	Simulator Run				
	II	III	IV	V	V-A
Thermocycle					
Max/min. °C	+20/-20	+20/-20	+20/-20	+20/-20	+20/-20
Hrs. ≧ 0°C	10	10	10	12	12
Hrs. at min.	12	12	12	10	10
Photocycle					
Intensity, ft.-cdls.	250	250	250	250	250
Spectral Character	Warm White Fluoresc.	Warm White Fluoresc.	Warm White Fluoresc.	Warm White Fluoresc.	Warm White Fluoresc.
Period, hrs.	12	12	12	16	16
Atmosphere					
N_2%	> 99.9	> 99.9	> 99.9	97	97
O_2%	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
CO_2%	< 0.1	< 0.1	< 0.1	3	3
H_2O, D.P., °C	-60	-60	-60	-60	-60
Flow Rate, L x min^{-1}	4	4	4	4	4
Surface					
Substratum	Perlite	Perlite	Perlite	Perlite	Perlite
Water gm/cm^2	0.3 (ice)	0.15 (ice)	0.3 (ice)	0.3	soaked
Frequency	2 wks.	2 wks.	2 wks.	4 wks.	seed
Species	Winter rye, peanuts, cucumber, celosia, corn	Winter rye, Morning Glory peanut	Winter rye	Winter rye	Winter rye
Special Conditions	None	Some of rye soaked in Quinones	First ice layer 0.5 M in KNO_3	Some seeds presoaked anaerobically in 0.01 M KNO_3	Test of glycine, ATP, KNO_3 effects

Table 2b

Experimental Results

Simulator Run No.	Period of Run (Days)	Species	Responses	Remarks
II	28	Rye	Germination 39% Root 13.5 mm Shoot 6.9 mm	Celosia should have succeeded, but very small seedlings desiccated. Other species affected mainly by freezing
		Corn	Germination 65% Root 3.1 mm No shoot	
		All other species failed		
III	14	Rye	1% Germinated	Peanut failed to germinate. Morning Glory root tips dried out.
		$+10^{-6}$M p-Benzoquinone		
		2,5-dichloro-	36%	
		2,5-dihydroxy-	21%	
		Morning Glory	62% Germination, then died.	
IV	7	Rye	1st shoots above ground	KNO_3 has doubled rate of Run No. II - seedlings comparable to Run II in size
	14		39% Germinated and with green shoots	
V	14	Rye	Emergence comparable with Run IV.	Without additional water, but with CO_2 and KNO_3, the best performance to date recorded.
	28		45% of Rye emerged with 20-25 mm shoots; 15-20 mm heavily branched roots.	
V-A	10	Rye	a. Water 0% Germ. b. 0.05 M Glycine 10 c. 0.01 M KNO_3 10 d. 0.01 M ATP 5 b + c 15 b + c 35 c + d 10 b + c + d 45	

supports the concept of intra-cellular O_2 production; however, it may reflect buildup of metabolites in a high enough state of oxidation to serve as electron acceptors (organic acids, for example).

In addition to the higher forms, we have unintentionally screened a variety of fungi in the simulator. These organisms were isolated from macroscopic (up to 1 cm^2) growths on or around young seedlings. The seedlings were not necessarily infected, hence the fungi may have grown on leachates from the seed. Some have been identified as follows:

Environment	No. of forms Distinguished	Forms Identified
Anaerobic Desert	at least 2	Mucor mucedo
Mars II	at least 4	Aspergillus niger
		Botrytis sp. (tentative)
		Torula sp. (yeast)
Mars III	at least 3	None
Mars IV	at least 5	Aspergillus sp.
		Aspergillus niger
		Torula sp.
Mars V	at least 4	Aspergillus sp.
		Botrytis sp. (tentative)

IV. HYDROGEN METABOLISM IN HIGHER PLANTS

The metabolism of H_2 by bacteria has been reported by many investigators (See Gest, 1954, Bact. Rev. 18, 43). Gaffron has described H_2 utilization by green algae, Kurata has reported the formation of hydrogenase during the ontogeny of the frog, and Sanadze has demonstrated H_2 evolution and uptake by illuminated leaves. As a result of in vitro studies on the evolution of H_2 by isolated chloroplasts, Boichenko has postulated the existence of hydrogenase in some higher plants.

The dependence of H_2 metabolism in bacteria and algae on the presence of hydrogenase, and the effects of inhibitors have been studied in detail by Fromagot, Gaffron, Valentine and others. Tagawa and Arnon have reported the isolation of a protein, ferredoxin, from the chloroplast of spinach leaves. This electron carrier, normally functioning in photosynthesis, can mediate both the reduction of pyridine nucleotide by H_2, and the production of H_2 in the presence of an electron donor, but only in the presence of added bacterial hydrogenase. While the identification of hydrogenase in higher plants has hitherto been uncertain its widespread occurrence in many different microorganisms, and its presumably important role under the reducing conditions of the primitive Earth, suggest that a latent, normally inactive hydrogenase system may be present in many higher organisms.

In the course of our studies on the effects of low O_2 tension on plants, we have reported the production of CO and other unusual metabolic products by Winter rye seedlings. We have also observed that Winter rye germinates more rapidly under various experimental atmospheres than under anaerobic conditions. This effect is most striking in CH_4, CO, and N_2O. As a result of finding free H_2 among various metabolic products, the following investigations were carried out to determine both the source of H_2 production and the effect of H_2 on seed germination.

A. Materials and Methods - The seeds used (Table 3) were incubated with 1 ml. H_2O in 10 ml. Erlenmeyer flasks sealed under air or experimental atmospheres with rubber septum caps, and maintained for several days under laboratory conditions of light and temperature. The seeds were generally pretreated in various ways to destroy bacteria, and all apparatus was sterilized in an autoclave. Gas samples were periodically removed by syringe and analyzed for H_2, O_2, and CO_2 by gas chromatography, using either a silica gel or a Linde "Molecular Sieve" (Type 5A) column at room temperature.

Bacterial counts were carried out by removing liquid samples, diluting with sterile H_2O, and spreading on petri dishes containing a solid agar medium with 17.5 g/liter Penassay Broth and 5 g/liter glucose. Colonies were counted after 20 hours incubation at 27°C.

Germination studies on Winter rye were carried out under various gas mixtures using 20 to 50 seeds on moist filter paper in disposable petri dishes. These were placed in 4 liter "anaerobic jars" as described by Siegel and Rosen. Each day the jar atmospheres were analyzed for O_2 using the Beckman D2 Paramagnetic Oxygen Analyzer, then were flushed with the appropriate experimental gas. The jars were stored under laboratory conditions, and germination counts made at daily intervals. Germination was based on the first visual radicle emergence. The gases were obtained from Linde Co., impurities being $< 0.1\%$ for H_2 and argon and $< 0.3\%$ for N_2.

The effect of metabolic inhibitors was studied using the above incubation arrangement and wetting the filter paper with a buffered solution of inhibitor. Controls were carried out using buffer alone.

The quoted results are generally averages of four independent experiments, each with 20 to 50 seeds.

B. Results - Non-sterilized Seeds: Hydrogen production by non-sterilized seeds is indicated in Table 3. Winter rye was studied in most detail, and evolved H_2 both in the dark and in light. Contamination of the seeds by H_2-producing

Table 3

H_2-Production in Seeds, Without Pretreatment

Family	Species	Common Name	H_2 Production
Liliaceae (Lily)	Allium Cepa, L.	Onion	-
Scrophulariaceae (Fig wort)	Antirrhinum majus, L.	Snapdragon	-
Cruciferae (Mustard)	Arabidopsis Thaliana, (L.) Heynh. var. Estland	Arabidopsis (cress)	-
Liliaceae	Asparagus officinalis, L.	Asparagus	-
Compositae (Composite)	Aster tanacetifolius, HBK.	Aster (Daisy)	+
Cruciferae	Brassica Rapa, L. "Purple Top White Globe"	Turnip	+
Amaranthaceae (Amaranth)	Celosia argentea, L. "Fire Feather"	Celosia	+
Labiatae (Mint)	Coleus Blumei, Benth.	Coleus	-
Convolvulaceae (Convolvulus)	Convolvulus japonicus, Thunb. "Blue Star"	(Calif. Rose) Morning Glory	+
Cucurbitaceae (Gourd)	Cucumis sativus, L. "Black Diamond"	Cucumber	+
Caryophyllaceae (Pink)	Dianthus barbatus, L. "Seven Sisters"	Sweet William	+
Papaveraceae (Poppy)	Eschscholtzia californica, Cham.	Calif. Poppy	+
Compositae	Helianthus annuus, L. "Giganteus"	Sunflower	+
Gramineae (Grass)	Hordeum vulgare, L.	Barley	+
Leguminosae	Glycine Max, Merr.	Soy Bean	+
Compositae	Lactuca sativa, L. "White Boston"	Lettuce	+
Solanaceae	Lycopersicum esculentum, Mill. "Manalucie"	Tomato	-
Labiatae (Mint)	Mentha piperita, L.	Peppermint	-
Portulacaceae	Portulaca grandiflora, Hook.	Portulaca (Purslane)	+
Cruciferae (Mustard)	Raphanus sativus, L.	Radish	+
Labiatae	Salvia splendens, Sello	Sage	+
Gramineae	Secale cereale, L.	Rye (Winter)	+

bacteria was confirmed by culturing the liquid phase from the seeds on solid Penassay medium. Such cultures obtained from different species of seed appeared identical, and formed moist, shiny, pale yellow, opaque colonies, with a smooth circular outline. Bacterial counts of the ambient liquid showed that a maximum concentration of 10^9 cells per ml., was reached after 3 days' incubation of the seeds.

When the bacteria were cultured in liquid Penassay Broth medium only traces of H_2 were evolved, but the addition of 20 autoclaved seeds increased H_2 evolution to concentrations similar to those obtained from untreated seeds germinating in H_2O (Table 4).

Seed Sterilization: The following treatments have been used in unsuccessful attempts to obtain sterility of Winter rye and other seeds:

I. 0.5% NaOCl, with shaking for 20 minutes, followed by four H_2O washes.

II. 5% NaOCl, with shaking for 1 minute, followed by four H_2O washes.

III. 0.01% $HgCl_2$, with shaking for 15 minutes, followed by four H_2O washes.

IV. Organo mercury compounds, e.g. Cerasan and Arasan.

V. Antibiotic mixture containing mycostatin (5,000 units per ml.), penicillin G (10,000 units per ml.) and streptomycin sulphate (1%).

Only the last treatment appeared to prevent bacterial growth, but no H_2 was evolved. However, in this case the viability of the seeds was also considerably reduced.

An apparently successful technique was developed using a dip in 70% ethanol followed by vigorous shaking for 20 minutes in 1% NaOCl and then four H_2O washes. Seeds treated in this way were put in 10 ml. Erlenmeyer flasks (one per flask) containing White's medium in 1% agar, supplemented with sucrose and A-Z micro-nutrient. All operations were carried out in a sterile transfer room. These flasks were plugged with cotton and incubated under ambient conditions of temperature, light, and atmosphere for 14 days. Then they were stoppered with sterile serum caps.

Table 4

Hydrogen Production by Winter Rye Seedlings and by Bacteria

No. of Days	% Composition by Volume in 10 ml. gas							
	Germinating Seeds in H_2O*		Bacteria in Penassay Broth					
			Alone				+20 Autoclaved Seeds	
	In Air		In Air		In N_2		In Air	
	H_2	O_2	H_2	O_2	H_2	O_2	H_2	O_2
Initially	0	20.9	0	20.9	0	0	0	20.9
1	0	17.0	0	13.0	0	1.2	0	12.5
2	0.2	1.5	0.2	8.5	0	1.2	0.4	1.4
3	0.5	1.4	0.3	7.0	0	1.5	1.2	1.0
4	1.3	1.0	0.3	6.5	0	1.4	1.6	0.9
5	4.1	0.5	0.3	4.5	0	1.7	2.4	0.9

* 20 unsterilized seeds sealed initially in air.

Sterilized Seeds: After sterilization, seedlings were cultivated in air in the complete absence of observable bacterial growth. Four days after sealing of the flasks the gas was analyzed and hydrogen was found from all four species tested (Table 5). Hydrogen production from sterilized seeds is enhanced by preliminary germination in air, followed by sealing under air after 1 to 4 cms. of shoot growth is obtained. The absence of bacteria in these samples was confirmed by (a) sterile transfer of seed coat and seedling to a petri dish containing Penassay Broth in agar, and smearing of the seedling across the surface before incubation for several days at 27°C, or (b) transfer of seedling to liquid Penassay medium and incubation for several days, followed by transfer of a drop of the liquid medium to a solid Penassay medium.

In no case was any bacterial growth found, proving that the H_2 production is attributable solely to the seedlings.

Germination and Growth: Under laboratory conditions of light and temperature, seeds of Winter rye (Secale cereale) showed enhanced germination under an atmosphere of H_2, compared with N_2 and argon respectively. The stimulatory effect of increasing amounts of H_2 in Ar is shown in Table 6. The continuing viability of seedlings on return to air after up to two weeks in these atmospheres was confirmed by their subsequent normal development.

Effect of O_2: Traces of O_2 have markedly different effects on germination of rye in H_2 and Ar. Jars containing Winter rye seeds that had been incubated for 21 hours under H_2 and Ar were injected with O_2. Germination counts after a further 24 hours showed that O_2 in concentrations $\leq 0.5\%$ stimulates germination in Ar, but inhibits in H_2 (Table 7).

The effects of some metabolic inhibitors on the germination of seeds under air, H_2, and argon are shown in Table 8. Because of the preliminary nature of this experiment, and its obvious extension to other inhibitors such as CO, no attempt is made to interpret the results in detail, but they do indicate alterations in metabolic pathways under these different atmospheric conditions.

Table 5

H_2 Production by Bacteria-free Seedlings, Germinated in Air, then Sealed for Four Days

Species	H_2 conc.	H_2 vol. in µl.
Radish (Raphanus sativus)	0.1%	6
Winter Rye (Secale cereale)	0.15%	12
Cucumber (Cucumis sativus)	0.2%	16
Turnip (Brassica rapa)	0.3%	24

Table 6

Germination of Winter Rye Seeds in Various Atmospheres

Composition of Atmosphere*	% Germination		
	1 day	2 days	3 days
Single gas			
Air (control)	95	95	95
H_2	15	45	55
Argon	10	20	20
N_2	0	10	20
H_2/argon mixtures			
100% H_2	18	35	45
25% H_2 in Ar	20	30	47
10% H_2 in Ar	20	20	40
5% H_2 in Ar	15	20	30
2.5% H_2 in Ar	5	15	30
100% Ar	10	20	23

* $O_2 < 0.1\%$

Table 7

Winter Rye Germination in Traces of O_2 after 21 Hrs. in H_2 or Argon

Gas	% Germination at 21 hrs.	% O_2 added at 21 hrs.	% Germination at 2 days	Change due to O_2
H_2	16	0	70	0
Ar	9	0	54	0
H_2	16	0.25	44	-37%
Ar	9	0.25	60	+11%
H_2	16	0.5	65	- 7%
Ar	9	0.5	80	+48%

Table 8

The Effect of Metabolic Inhibitors on Winter Rye Germination

Inhibitor	Conc. M	% Inhibition after 3 Days		
		Air	H_2	Argon
KCN	10^{-2}	> 80	14	29
NaN_3	5×10^{-4}	8	47	39
Dinitrophenol	5×10^{-4}	5	66	69
NaF	5×10^{-4}	5	11	64

C. Discussion - The seeds of 15 species are found to be contaminated with H_2-producing bacteria which are highly resistant to sterilization. The results show the importance of rigorous sterilization techniques in the in vivo and in vitro determination of hydrogenase activity, and indicate that some procedures commonly used for seed sterilization may be far from adequate. The bacteria evolve H_2 only under near anaerobic conditions, and show an interesting dependence upon seed constituents for maximum H_2 production.

Since H_2 production and assimilation are generally assumed to depend on the presence of a hydrogenase system, the evolution of H_2 by bacteria-free seedlings in closed systems is presented as evidence for the occurrence of hydrogenase in these plants.

The positive response of Winter rye seeds under pure H_2, and the inhibition of germination by small amounts of O_2, as compared to stimulation by O_2 in similar tests under Ar, show an altered metabolism, possibly arising from a partial adaptation to H_2, and is additional evidence for the existence of hydrogenase.

Demonstration of H_2 uptake by sterilized seeds and seedlings in a Warburg respirometer has not yet been successful, even in the presence of the commonly used electron acceptors, methylene blue and methyl viologen, although reduction of the latter to the colored state was obtained.

In the H_2-production experiments, the very small amounts of gas evolved under sterile conditions indicate either a very low hydrogenase concentration or lack of some cofactor requirement, either of which may cause difficulty in determining hydrogenase activity by the normal methods.

V. GENETIC AND CYTOLOGICAL STUDIES: PRELIMINARY EXAMINATION OF GRASSES AND LEGUMES

Screening for anaerophilic plants has concentrated on two families of angiosperms which include many of the more important crops - Graminae (cereals, forage grasses) and Leguminosae (beans, peas, vetch, clovers). Seed germination under anoxia has been used as the criterion of anaerophilia. Seeds from representative species in each family were enclosed in lucite pressure chambers which were then evacuated and filled with air (control), hydrogen, or argon. The limited space within the chambers used to date necessitated the use of relatively small samples of seeds, particularly in the case of the larger seeded legumes. Survival as well as germination was recorded to assess possible deleterious effects of the treatments.

Some preliminary results with Gramineous species are summarized in Table 9. The self-pollinated cereals (Barley, Spelt, Oats), except for Genessee Wheat, showed little capacity for anaerobic germination. Conversely, seed samples from three varieties of the cross-pollinated species, Winter rye included sizeable numbers of seeds which germinated under anoxia. Intervarietal differences were evident from the significantly greater proportion of "anaerobes" in the population from 3915.

Because of its ability to germinate under anaerobic stress, Winter rye was used as a reference standard in subsequent experiments. In those experiments summarized in Table 10 the two varieties of rye showed relatively high and approximately equal proportions of "anaerobes". However, these frequencies were greatly exceeded by those characteristic of the self-pollinated species, rice. The variety Calaro, a relatively cold resistant, japonica type of rice, included a greater proportion of anaerobes than did the indica type variety, Potna. Among those sorghums tested only the inbred variety Combine Kafir 60 showed a level of anaerobic germination comparable to that of rye. The hybrid varieties were included because of such special attributes as drought resistance (67 GX), cold tolerance (410E) and early maturity (209A and 109FX). Though their parentage was unknown the hybrids

Table 9

Effects of Anoxia on Germination of Cereal Grains

		% Germination (2-3 days)				% Survival			
Type	Variety	n	Air	Argon	% Control	n	Air	Argon	% Control
Barley	Erie	100	93	0	0	100	92	48	52 ←
Wheat	Genessee	150	92	25	28	150	97	90	93
Spelt	--	30	63	0	0	30	93	87	93
Oats	Victory	60	100	0	0	60	100	100	100
Oats	Wintok	30	90	0	0	30	93	100	107
Oats	Wild	80	100	0	0	80	100	38	38 ←
W. Rye	3915	150	97	34	35	150	97	81	84
W. Rye	816	150	89	19	21	150	93	77	83
W. Rye	R.P.	50	92	26	28	50	94	44	47 ←
W. Rye	Rosen	50	100	12	12	-	-	-	-

Table 10

Germination of Certain Gramineous Species and Varieties under Anoxia

Type	Variety	% Germination (3-4 days) n	Air	Argon	% Control	% Survival (7-8 days) n	Air	Argon	% Control
Sorghum (grain type)	Combine Kafir 60	50	92	60	65 <—	50	96	90	94
"	3058	50	100	2	2	50	100	98	98
Sorghum	Hybrid 410E	50	96	20	21	50	98	98	100
"	Hybrid 67GX	50	88	14	16	50	96	88	92
"	Hybrid 209A	50	94	44	47 <—	50	96	90	94
"	Hybrid 109FX	50	100	16	16	50	100	90	90
Rice	Calora	100	99	100	101 <—	100	99	100	101
Rice	Patna	100	89	74	83	100	90	84	93
Rye	3915	100	98	68	69	100	98	94	96
Rye	816	100	93	62	67	100	93	87	94

gave little evidence of heterosis for "anaerophilia". Only hybrid 209A showed a marked anaerobic potential and even this was less than that demonstrated by Combine Kafir 60.

Longer periods of germination under anoxia gave more clear-cut evidence of inter-varietal differences in anaerophilia (Table 11). The frequency of anaerobic seeds in the sample from inbred sorghum Combine Kafir 60 exceeded that from the hybrid 410E and was equal to that from variety #816 of Winter rye. Similarly the proportion of anaerobes in the seed sample from Winter rye variety 3915 was clearly greater than that in variety 816. Differences in survival of seed kept in argon (7 days) and the control gave little evidence of reduction in viability due to anoxia.

Previous work with legumes had shown that, except for the peanut (Arachis hypogea), members of this group possessed relatively low anaerobic potential. Though legumes as a group were clearly inferior in this respect to grasses, i.e., germination in argon was effectively nil, recent results have been more encouraging (Table 12). Germination of both broad bean and sieva bean was greater than that of peanut and comparable to that of Winter rye. Survival of these legumes was not impaired by hydrogen but tended to be reduced in argon.

Squash preparations were made from roots of Vicia faba seeds germinated in hydrogen and checked for dividing cells. Five of the fifteen germinating beans were examined and found to be mitotically active. Mitotic rates were less than those of the controls and the frequency distribution of mitotic stages suggested that these cells may have only recently become active (Table 13). All seven roots of sieva bean (100% germination) examined were found to be mitotically active. Four of eleven peanuts germinated. Two had roots of sufficient length to make mitosis probable but only one of them was mitotically active. These data suggest that both broad bean and sieva bean are better anaerobes than the peanut though the possibility that rate differences in germination influenced the comparisons cannot yet be excluded.

Table 11

Intervarietal Differences in Anaerobic Germination of Sorghum and Winter Rye

Type	Variety	% Germination (7 days) n	Air	Argon	% Control	% Survival (12 days) n	Air	Argon	% Control
Sorghum	Combine Kafir 60	100	92	38	41	100	92	79	86
"	Hybrid 410E	100	92	9	10	100	92	95	103
W. Rye	3915	100	99	53	54	100	99	95	96
W. Rye	816	100	93	37	40	100	93	81	87

Table 12

Anaerobic Germination of Representative Legumes

Species	% Germination (4-8 days) n/tr	Air	H_2	Argon	% Survival n/tr	Air	H_2	Argon
Vicia faba (Broad Bean)	28	86	96	0	16	93	93	55
Vicia villosa (Winter Vetch)	100	78	14	0	100	83	86	71
Arachis hypogea (Peanut)	23	100	65	0	11	100	100	80
Pisum sativum (Garden Pea)	30	20	3	0	30	87	87	100
Phaseolus henatus (Sieva Bean)	16	100	100	6	16	100	100	88
Secale cereale (Winter Rye) 3915	100	97	91	73	100	97	88	72

Table 13

Effect of Environment on Mitosis in Roots of Vicia faba

Treatment	$\bar{x}$ Root Length (mm)	Mitotic Cells: Interphase Cells	Pro-phase	Meto-phase	Ana-tetophase	Total Cells	Mitotic Cells	M.I.
Control	44	3297	195	31	75	3598	301	8.5
H_2	10	3562	22	13	3	3600	38	1.1

Table 14

Anaerobic Germination of Phaseolus Species and Varieties

Species	% Germination (6-7 days) n/tr	Air	H_2	$\bar{x}$ Root Length	% Survival (11 days) n/tr	Air	H_2	Argon
Phaseolus limensis (Bush Lima Bean)	30	100	70	60 mm.	21	100	76	
Phaseolus lunatus (Sieva Bean)	30	100	77	89 mm.	18	100	83	
Phaseolus vulgaris (Pole Bean)	40	100	0	83 mm.	39	100	77	
Phaseolus vulgaris (Bush Bean)	40	100	70	97 mm.	29	100	63	
Phaseolus vulgaris (Shell Bean)	40	98	55	96 mm.	30	98	67	

None of the species examined cytologically were able to initiate mitosis in argon although members of other families can do so. This was surprising, particularly in the case of Winter rye, which germinated readily in argon and showed a relatively high level of mitotic activity in hydrogen (Latterell, unpublished). Closer investigation revealed that "germination" recorded in argon involved expansion of the coleorbiza but no commensurate elongation of the enclosed root. Evidently cell enlargement and cell division show differential suppression in argon. This finding calls into question the utility of argon as a medium for selecting plants with anaerobic potentialities. The possibility of using other gases or mixtures of gases is being investigated.

The relatively vigorous growth of sieva bean under anoxia prompted a comparison of this species with other available types of Phaseolus. In this instance differences between sieva bean and other members of the genus were less striking than anticipated. Interspecific and intervarietal differences were demonstrated, however, most striking of which was the complete inability of the pole bean to germinate under anoxia. Control roots for this type and the other two varieties of Phaseolus vulgaris tested showed comparable amounts of root elongation (Table 14, column 5).

Summary

These studies have provided evidence for interspecific and intervarietal differences in the ability of seeds to germinate under anaerobic conditions. Cytological studies have provided indications as to what extent and under what conditions components of developmental systems are suppressed by anoxia. Some more detailed genetic and cytological studies are planned for the more anaerophilic self-pollinated species, Rice, Wheat and Sorghum. Similar work will be conducted with Winter rye in view of its relatively great resistance to anaerobic stress and the general interest in comparisons of self- and cross-pollinated plants. In this connection, investigations of anaerobic abilities of certain cross-pollinated legumes, specifically red and white clover, are being considered.

VI. THE USE OF OXIDANTS AND OXIDIZING CONDITIONS AS PARTIAL SUBSTITUTES FOR O_2

This section is concerned with methods whereby seeds may be induced to germinate and produce seedlings under adverse atmospheric conditions.

Seed germination is commonly regarded to be an aerobic process. Although some seeds, rice for example, can germinate under water - and even in environments containing no O_2 - most seeds require some O_2 to be present.

During a study concerned with interactions between ionizing radiation and O_2 in germination and seedling growth, it was surprising to observe that the ionization of an oxygen-free atmosphere enabled the atmosphere to support germination. Ionizing radiation is known to produce oxidizing species in atmospheric gases; in various liquids including water; in various solutions, including aqueous solutions; in biological fluids; and in biological materials, both wet and dry. These oxidizing species include: H_2O^+; HO^+; $HO\cdot$; N_2^+; H_2O_2; ROOH, wherein R is any one of many organic moieties; RO^+; $RO\cdot$; and other oxidants both of ionic and free radical character. Such oxidants may act directly upon an O_2-limited process essential to growth; alternatively, they may oxidize or raise to a higher oxidation number or valence, some one or more cellular constituents, such as coenzymes, metal ions, or metal ion-complexes.

It is also possible for some but not all of these oxidants to give rise to O_2 itself via biochemical or purely chemical means.

In the ordinary pursuits of gardening, agriculture, and horticulture, the preparation of seed beds should provide suitable amounts of oxygen for the development of seedlings. There are many instances, however, in which seeds fail to germinate, are delayed in germinating, or produce defective seedlings in soils that are excessively dense - in clays for example; in lowland areas having a water table near the surface; in poorly drained upland areas - those overlying shale or other impervious strata; and in swamps, bogs, and marshes.

Furthermore, in any future efforts to cultivate plants under artificial conditions in closed systems, including orbital and interplanetary vehicles, and in manned ground installations elsewhere than on Earth, it may be desirable to render plant growth independent of molecular oxygen or of conventionally stored oxygen. Conceivably, aqueous solutions prepared from relatively stable solid or liquid oxidants, or aqueous solutions (of nutrients, for example) which have been exposed to ionizing radiation, could be used directly as media within which to germinate seeds and maintain plants. These plants would thereby be rendered independent of an atmospheric oxygen supply, allowing for great economy in the materials, efforts, and expense of artificial, non-terrestrial plant production for food or other purposes.

Experimentally, one procedure studied consisted of incubating seeds in water or moist substrata such as absorbent paper, diatomaceous earth, perlite, or vermiculite, with a moist atmosphere which contained a suitable energy source for ionization. Because there is a diversity of forms and sources of energy, the level of energy and concentration of ions are not the most convenient reference values for establishing the effective treatment. Instead, it is recommended that the ionization be expressed in equivalents of oxidizing power, namely, as equivalents of hydrogen peroxide which may be determined by standard volumetric analysis. On that basis, ionizations which provide approximately 2×10^{-5} moles of oxidants as H_2O_2 per seed are recommended, within the range of 5×10^{-6} to 5×10^{-5} moles/seed. Said amounts of oxidant are assumed to be the integrated supply during a 1-3 day period of incubation.

A second procedure consists of incubating seeds in water or aqueous solutions of inorganic nutrients after said water or solution has been subjected to radiolysis. It is well known that water or water solutions, as well as water vapor, form H_2O_2 and other oxidizing species upon exposure to ionizing radiation.

The third and most useful incubation procedure made use of solutions containing suitable oxidizing agents. Useful concentrations of oxidants in such solutions vary, and will be specified below.

For our purposes, a number of oxidants can be employed, including H_2O_2, hydroperoxides, peroxides, quinones, and some inorganic oxidants.

Obviously, this array of compounds differs in many ways, hence a single concentration (and concentration range) cannot be prescribed which applies to all classes. In the following table, however, appropriate concentrations are summarized:

Substance(s)	Concentration (moles/liter)
H_2O_2	0.02-0.2
all other hydroperoxides	10^{-5} to 10^{-4}
all peroxides	10^{-5} to 10^{-4}
all quinones	5×10^{-7} to 2×10^{-6}
sulfur	seeds are planted in wet sulfur
other members of periodic group VIa	0.005-0.05
other members of Class III	10^{-4} to 5×10^{-3}

Some peroxygen compounds cannot be employed directly to contact the seed or plant because they are active oxidants of essential cellular structures and/or components. Organic peracids such as peracetic, and inorganic peroxyacids such as peroxydisulfuric, are excluded upon such grounds. Peroxyboric acid is excluded because boric acid derivatives are toxic save as trace additions to plant nutrients; peroxymolybdic acid and other peroxy acids derived from heavy metals are also to be avoided for reasons of toxicity. Metal peroxides and superoxides such as BaO_2, KO_2 and Na_2O_2 are commonly to be avoided because they generate toxic heavy metal ions or caustic products in water. Among these substances, however, are those which will generate H_2O_2 under suitable conditions, for example, $BaO_2 + H_2SO_4 \longrightarrow BaSO_4 + H_2O_2$.

Such substances should not contact the seed directly, but could be used to generate H_2O_2 which is, in turn, delivered to the seed or plant in gaseous or liquid form.

In the examples which follow, the effects of representatives of the several classes of oxidants upon various species are summarized.

Example 1 - Quinones

Turnip and cucumber seed in groups of 25-50 were placed on filter paper moistened with water or aqueous solutions of various quinones under an atmosphere consisting of 5% O_2 + 95% argon. After time intervals as specified, germination and growth data were obtained.

A. Turnip Germination in 10^{-6} Molar Solutions

Composition	Germination Percentage After	
	2 Days	3 Days
water	6	33
p-benzoquinone	28	48
2,5-dihydroxy-p-benzoquinone	10	60
2,6-dichloro-p-benzoquinone	27	56
2,5-di-tert-butyl-p-benzoquinone	29	54

B. Turnip Radicle Emergence in 10^{-6} Molar Solutions

Composition	Radicle Length in mm. at 6 Days
water	2.6
p-benzoquinone	5.0
2,5-dichloro-p-benzoquinone	6.0
2,6-dichloro-p-benzoquinone	5.0
2,5-di-tert-butyl-p-benzoquinone	8.6
2-tolu-p-benzoquinone	10.0
2-methyl-1,4-naphthoquinone	6.4

C. Cucumber Germination in 10^{-6} Molar Solutions

Composition	Germination Percentage at 2 Days
water	54
2,5-dichloro-p-benzoquinone	84
2,6-dichloro-p-benzoquinone	68
2,5-dihydroxy-p-benzoquinone	69
2,5-di-tert-butyl-p-benzoquinone	61

D. Cucumber Seedling Weight in 10^{-6} Molar Solutions

Composition	Fresh Weight in mgm at 6 Days
water	71
2,6-dichloro-p-benzoquinone	83
2,5-dihydroxy-p-benzoquinone	89
2-methyl-1,4-naphthoquinone	88

The foregoing results show that some quinones can be particularly effective as stimulants of germination and growth when oxygen is supplied in limiting amounts.

Example II - Inorganic Oxidants-General

Seeds of lettuce, Winter rye and cucumber in groups of 50 or more were incubated in water or aqueous solutions of inorganic oxidants under an Argon atmosphere containing less than 0.1% O_2. Germination counts were made at specified intervals of time.

A. Lettuce

Composition	Germination Percentage after 7 Days
water	0.0
10^{-4}M Ceric Sulfate	6.6
10^{-4}M Sodium Dichromate	4.0
10^{-3}M Potassium Ferricyanide	5.0

B. Effect of Nitrate and Tellurite on Three Species

Species	Incubation Time	Germination Percentage in Water only	Germination Percentage in 2.5×10^{-3} Potass. nitrate	Germination Percentage in 10^{-2}M Potass. Tellurite
Lettuce	7 days	0	8	-
Rye	5 days	37	55	62
Cucumber	4 days	20	40	59

C. Effect of Sodium Percarbonate on Turnip Germination

Treatment	Germination Percentage in 40 Hrs.
water	0
0.05 M Na_2CO_4	8

These results show several inorganic oxidants, both cationic and anionic, to be at least somewhat effective as stimulants of germination under near-anaerobic conditions.

Example III - Elementary Sulfur

Nine volumes of flowers of sulfur, one volume of 3-8 mesh silica gel and 6 volumes of water were combined and mixed manually until a homogeneous paste was formed. Seeds of lettuce, turnip, cucumber and Celosia in groups of 30-50 were placed on the sulfur paste or on filter paper, and incubated under 2% O_2- 98% argon or 5% O_2- 95% argon. Germination and growth measurements were made at intervals.

A. Germination of Several Species in Sulfur

Species	Germination Percentage After 5 Days under 2% O_2- 98% Argon		Germination Percentage After 2 Days under 5% O_2- 95% Argon	
	In Water	In Sulfur	In Water	In Sulfur
Lettuce	8	24	61	83
Turnip	16	24	21	43
Cucumber	50	81	16	64
Celosia			36	52

B. Growth of Cucumber Seedlings in 5 Days

Growth Measurement	2% O_2 - 98% Argon		5% O_2 + 95% Argon	
	In Water	In Sulfur	In Water	In Sulfur
Root Length, mm.	12.2	18.6	14.5	20.4
Number of Secondary roots/seedling	-	-	1.8	8.7
Fresh wt. mgm/seedling	56	73	65	92

These results demonstrate the novel and unexpected properties of elementary sulfur as a growth stimulant.

Example IV - Ionization

Winter rye grains were placed in concentric rings on a water-saturated perlite surface in a container 15 cm. in diameter. Air was removed by aspiration and the jars filled to 1 atm. pressure with inert gas or kept continuously on aspiration. In the former, N_2 or argon was used to fill containers; in the last instance. water vapor at approximately 0.5 psig constituted the only atmosphere. Although rye can germinate and grow in any of these atmospheres, its rate of development may be markedly increased by introduction of an ionizing source. For this purpose, strips of Po^{210} 5 cm x 1 cm (500 µcuries) were introduced into these oxygen-free atmospheres but so placed that little or no alpha-radiation would contact the seeds directly.

A. Four-500µcurie Po^{210} Strips Centrally Placed; Atmosphere of Nitrogen

		6-Day Growth Data		
Atmosphere	Germination Percentage	Ave. Root Length, mm	Ave. Shoot Length, mm	% Green Shoots
Untreated	75	15.6	10.8	44
Ionized	95	28.1	16.7	95

B. Four-500µcurie Po^{210} Strips Peripherally Placed; Atmosphere of Argon

		3-Day Growth Data		
Atmosphere	Germination Percentage	Ave. Root Length, mm	Ave. Shoot Length, mm	No. of Secondary Roots
Untreated	49	3.8	2.0	0.3
Ionized	87	7.0	2.6	1.1

C. Four-500μcurie Po^{210} Strips Peripherally Placed; Atmosphere of Water Vapor at 0.5 psig

	4-Day Growth Data	
Atmosphere	Germination Percentage	Root Length mm
Untreated	30	1.5
Ionized	73	6.5

The foregoing results show strikingly the stimulatory effects of ionizing radiation under anaerobic conditions.

Example V - Peroxygen Compounds

Seeds of rye, lettuce, and turnip in groups of 30-40 were incubated in argon atmospheres devoid of all but traces of oxygen. Rye can germinate and grow under this circumstance; neither lettuce nor turnip show signs of germination without oxygen at levels of over 1% by volume. When H_2O_2 or various other peroxygen compounds are supplied at appropriate levels, the germination of all three species is benefitted greatly.

A. Rye Germination under Argon in Hydrogen Peroxide Solutions

H_2O_2 Concentration		Germination Percentage	
Moles/L	Moles/Seed	One Day	Two-Days
0	0	15	44
0.01	0.2×10^{-5}	29	44
0.05	1.0×10^{-5}	44	67
0.08	1.6×10^{-5}	40	75
0.1	2.0×10^{-5}	54	96

B. Hydrogen Peroxide - Enhanced Germination of Oxygen Dependent Lettuce and Turnip in Argon

Species	Treatment	Germination Percentage After		
		16.5 hrs.	41 hrs.	5 Days
Lettuce	none	0	0	0
	0.05 M H_2O_2	7	48	70
	0.1 M H_2O_2	0	47	91
Turnip	none	0	0	0
	0.05 M H_2O_2	28	86	> 90
	0.1 M H_2O_2	43	89	> 90

C. Germination of Rye in Argon with Various Peroxides and Hydroperoxides

Peroxide	Concentration Moles/L	Germination Percentage After 16 Hrs.	21 Hrs.
none	-	4	15
Ethyl Hydroperoxide	5×10^{-5}	23	-
	5×10^{-4}	12	-
p-Menthane Hydroperoxide	10^{-5}	20	-
	5×10^{-5}	-	23
	5×10^{-4}	-	8
Methyl ethyl ketone peroxide	5×10^{-5}	8	-
	5×10^{-4}	8	-
Ascaridole	5×10^{-5}	21	-
	10^{-4}	-	24

VII THE EFFECTS OF ATMOSPHERE ON GERMINATION OF RYE IN SALT, NUTRIENT AND METABOLITE SOLUTIONS

A. Air Pressure, Calcium and Osmotic Pressure Relations - Winter rye incubated for 3 days at 25°C in air shows a regular decline in germination as the osmotic pressure of NaCl solutions is increased (Figure 3). The presence of calcium (as nitrate) enables rye to germinate without inhibition, even at the highest osmotic concentration tested.

A reduction of only one-third in air pressure renders the seeds more sensitive to osmotic inhibition, especially in the absence of Ca^{2+}.

At ca one-third atm., inhibition is still more pronounced than at higher air pressures, and the effect of Ca^{2+} is small.

At one-tenth atm., germination is severely expressed in salt solutions, and the presence of $Ca(NO_3)_2$ has little or no effect.

In water alone, reduction in P_{air} has no effect other than a slight reduction in germination rate.

The effect of atmospheres and solutions upon germination is expressed in part by the osmotic pressure required for 50% inhibition of germination (Table 15), which is appreciably higher in air when $Ca(NO_3)_2$ is present. Note, however, the convergence of the + calcium and - calcium series as air (and O_2) pressure declines.

Root growth decreases regularly with increasing O.P. in NaCl, but at lower salt concentrations exhibits a weak optimum as P_{air} is reduced (Figure 4). In the presence of $Ca(NO_3)_2$, however, root lengths are similar at O.P. = 0 and O.P. = 0.72, and only slightly depressed at O.P. = 3.6. Root emergence occurs even at O.P. = 21.6 except at P_{air} = 76 mm Hg when calcium nitrate is present, but not at any air pressure in pure NaCl.

P_{Air} = 76 mm Hg ~~P_{Air} = 290 mm Hg~~

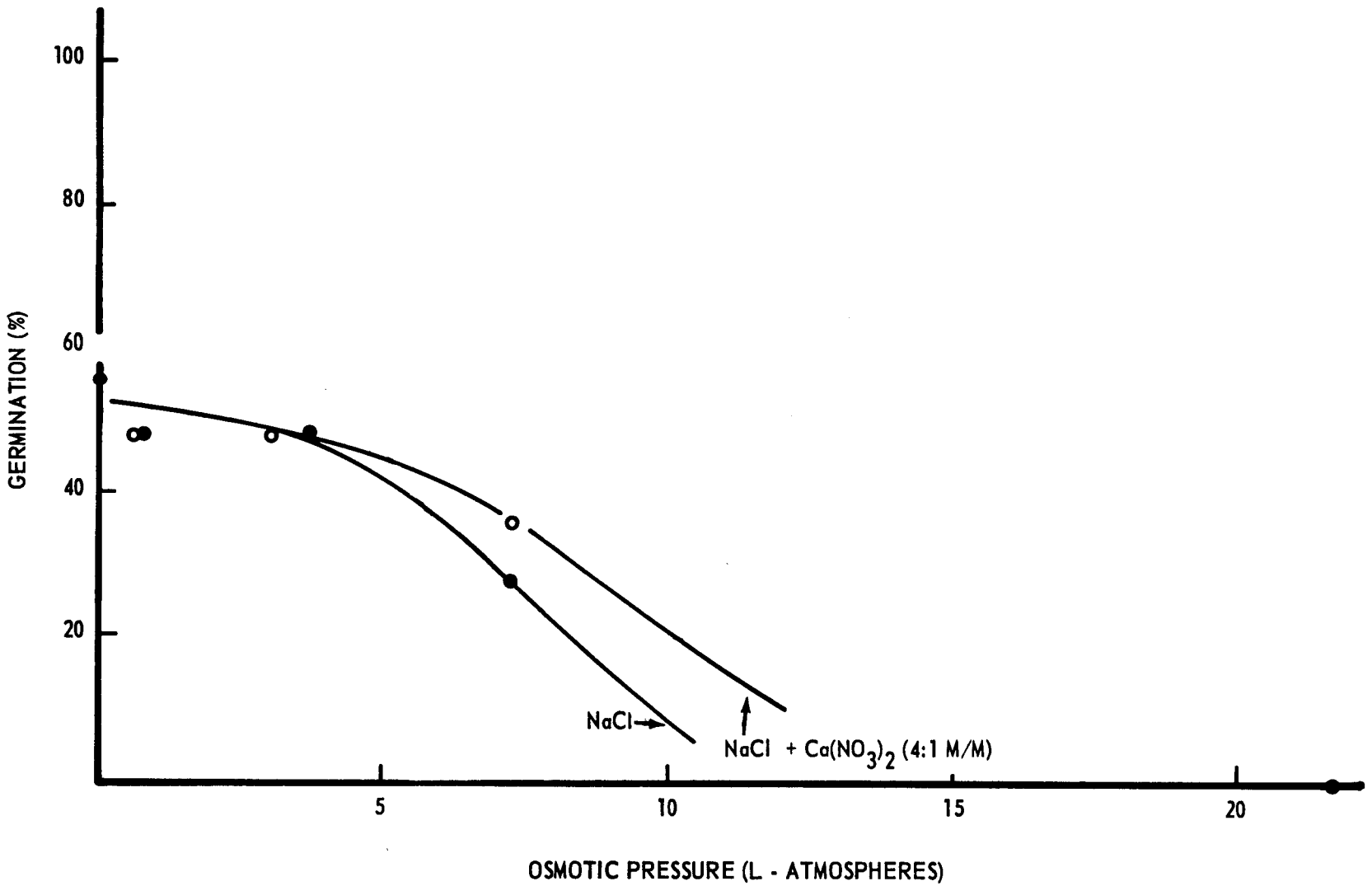
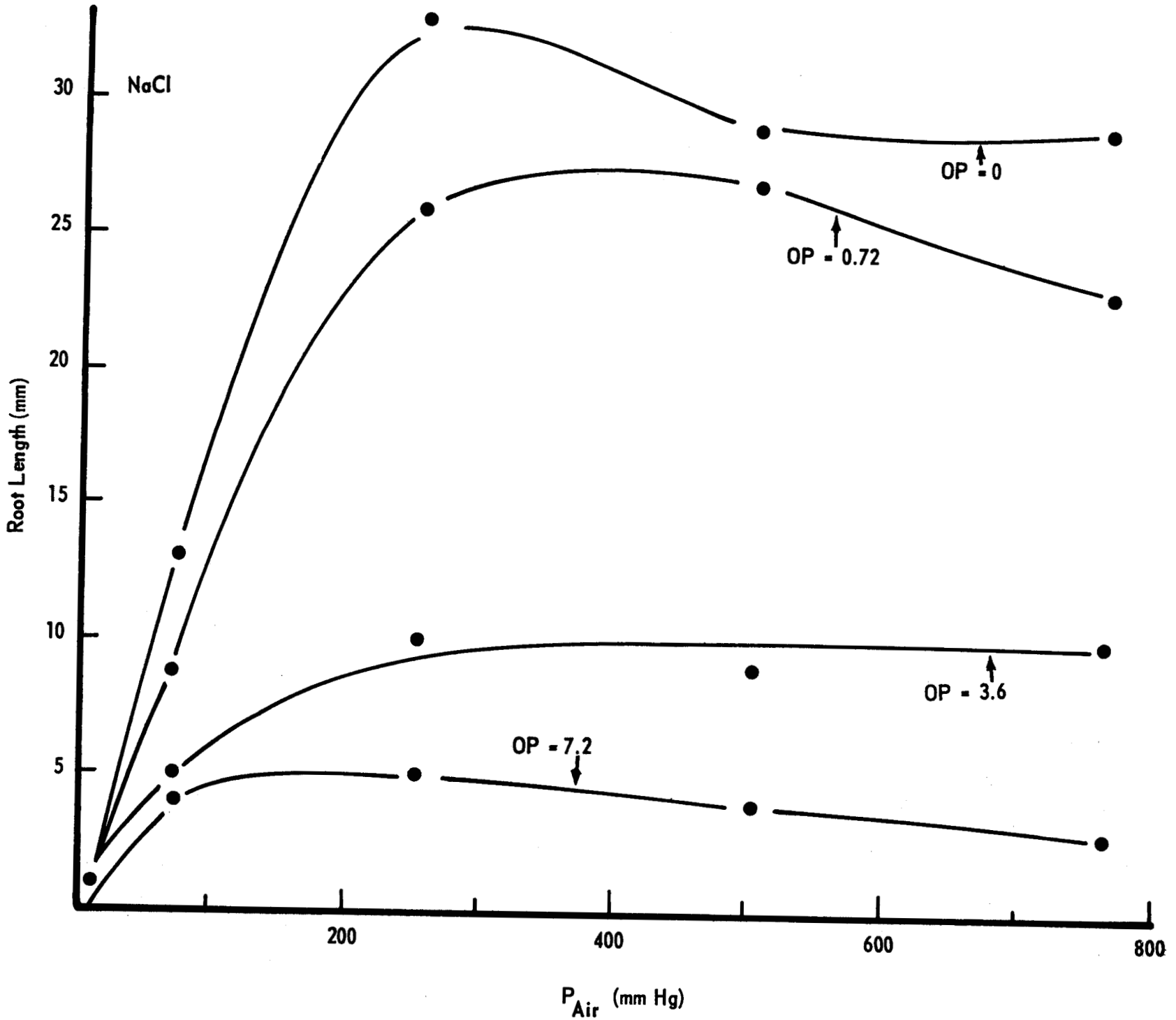


Table 15

Osmotic Pressures for 50% Inhibition of Rye Germination: Interpolated Values

P_{air}(mm)	Osmotic Pressure (L-Atmospheres) NaCl	NaCl + Ca $(NO_3)_2$ (4.1 M/M)
760	11.4	> 18
500	6.4	> 18
250	6.5	11
76	7.1	8.7
12	$\leq 3.6^*$	$\leq 3.6^*$

* This value is of uncertain magnitude as a result of variability in germination at very low pressure, hence only a maximum value is given.


NaCl
30
25
20
15
10
5
Root Length (mm)
OP = 0
OP = 0.72
OP = 3.6
OP = 7.2
200
400
600
800
P Air (mm Hg)

The effect of $Ca(NO_3)_2$ in reducing osmotic inhibition is not self-evident. The Ca-ion itself may serve a protective function by counteracting the effects of Na^+ on membrane permeability and cytoplasmic organization. Nitrate may serve as an electron acceptor when O_2 is limiting. Accordingly, the effects of $Ca(NO_3)_2$, $CaCl_2$, and KNO_3 on NaCl inhibition were compared (Table 16).

The data show that the effect of $Ca(NO_3)_2$ is complex, with contributions from both Ca^{++} and $NO_3^{=}$.

The effect of added salts on NaCl inhibition may be summarized as follows:

	Effective Ion	
P_{air}(mm Hg)	Germination	Root Length
760	Not inhibited	Ca^{2+}
250	Not inhibited	Ca^{2+} + NO_3^- only
76	Ca^{2+}, NO_3^-, both	Ca^{2+} + NO_3^- only
12	None	None

B. Atmospheres and Phosphate Compunds

Because of their significance in biological energy transfer mechanisms, phosphorous compounds were selected for the first of a series of studies seeking out chemical stimulants (other than quinones and peroxides which may have undesirable side reactions) of anaerobic germination.

Orthophosphate was first tested using a mixture of potassium and sodium (Table 17) (K^+/Na^+ = 1) salts with a primary-secondary ratio $H_2PO_4^-/HPO_4^=$ of 2 and pH = 7.0.

Relative to its own lowest concentration, 0.005 M, higher levels of phosphate enhance germination in several cases both aerobically and anaerobically (Fig. 5). This was noted with rye from two different sources (identified by supplier's name) which were genetically similar, but of different history.

Table 16

Relation of Atmosphere and Salt Combinations to

Germination and Seedling Growth

Solution	P_{air} (mm Hg)							
	760		250		76		12	
	Germ. (%)	Root (mm)	Germ. (%)	Root (mm)	Germ. (%)	Root (mm)	Germ. (%)	Root (mm)
Water	80	29	60	34	56	13	16	1
NaCl*	76	10	68	8	8	3	6	1
NaCl + Ca $(NO_3)_2$*	84	30	64	28	38	11	0	0
+ Ca Cl_2*	60	37	52	10	20	4	4	1
+ KNO_3*	84	11	64	17	28	3	0	1

*All at O.P. = ca 3.0-3.6 L-atm., NaCl: X Ca. 4:1

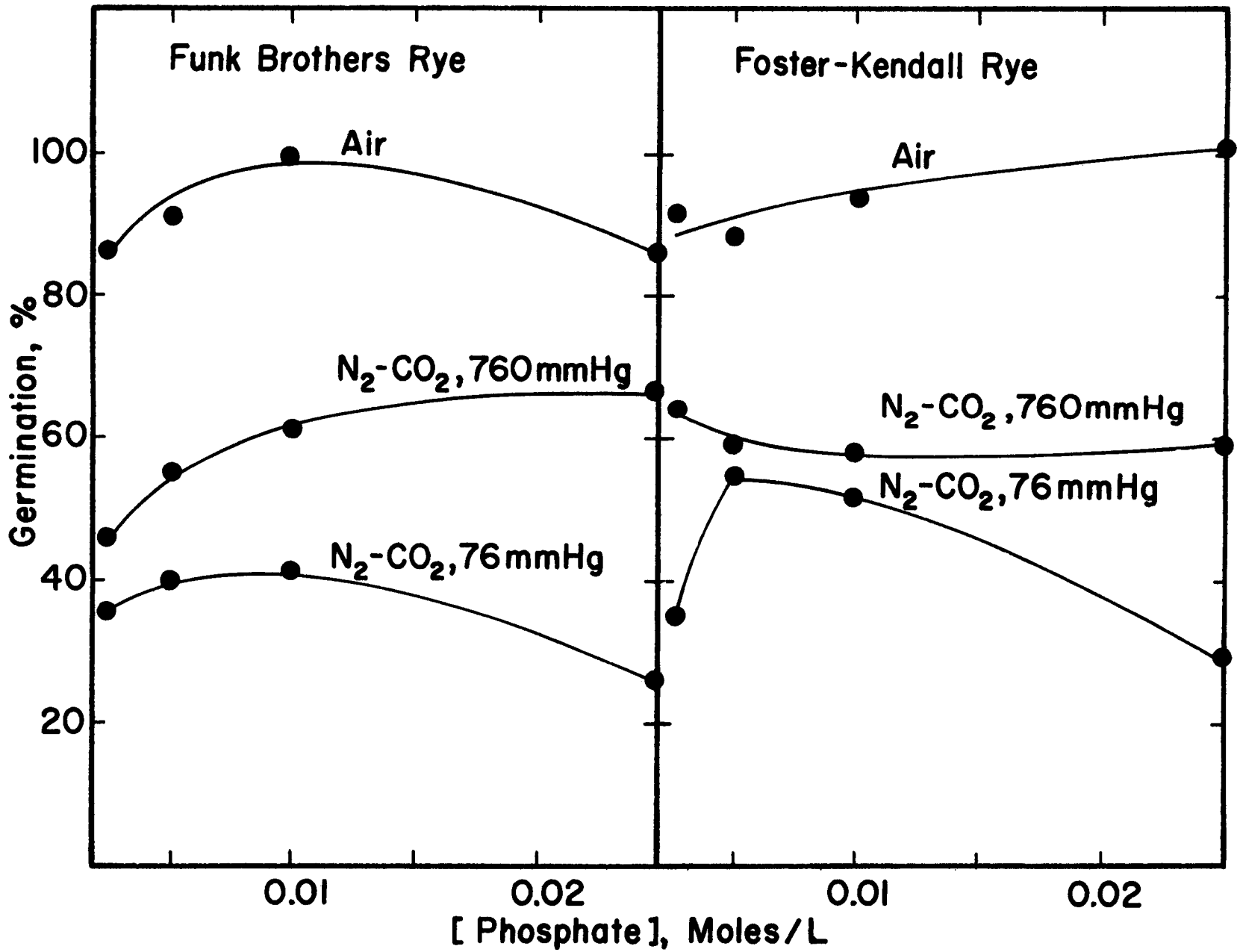
Table 17

Root and Coleoptile Lengths of Rye After

3 Days in Phosphate Solutions

Source of Seedstock: Funk Bros.

(Phosphate), M	Air		97% N_2 - 3% CO_2		97% N_2 - 3% CO_2	
	760 mm Hg		760 mm Hg		76 mm Hg	
	Root	Coleoptile	Root	Coleoptile	Root	Coleoptile
0.0025	37	35	4	4	5	5
0.005	26	35	4	4	9	9
0.01	35	41	4	4	12	7
0.025	20	32	4	4	6	6
Source of Seedstock: Foster-Kendall						
0.0025	56	36	5	4	11	9
0.005	54	35	5	4	7	6
0.01	37	35	5	8	9	6
0.025	37	35	5	4	10	5


Funk Brothers Rye
Foster-Kendall Rye
Air
Air
N_2-CO_2, 760mmHg
N_2-CO_2, 760mmHg
N_2-CO_2, 76mmHg
N_2-CO_2, 76mmHg
Germination, %
100
80
60
40
20
0.01
0.02
0.01
0.02
[Phosphate], Moles/L

In air, root growth was suppressed somewhat by higher phosphate concentrations, whereas coleoptile growth was not affected. These data are not yet adequate to give any clear understanding of the range of effects phosphate may have, but it indicates that the growth can be influenced by phosphate supply.

A number of organic phosphates have also been tested at 0.005 M, and compared with the same concentration of orthophosphate (Table 18). Some stimulate aerobically, none do so anaerobically. Others inhibit growth aerobically and anaerobically.

Table 18

Phosphate Compounds Tested on Winter Rye

a. Aerobic Stimulants*

Fructose-1,6-diphosphate

Glucose-phosphate

RNA Hydrolyzate

b. Anaerobic Stimulants*

None

c. Aerobic Inhibitors*

Inositol Phosphatide

Diethanolamine phosphate

d. Anaerobic Inhibitors*

Urethanolamine Phosphate

Fructose 1,6-d

Glucose-1-phosphate

RNA Hydrolyzate

Glucose-6-phosphate

* All relative to 0.05 M Phosphate

VIII. The Experimental Biology of Simulated Environments: Conclusions

Although environmental simulation has been practiced by many investigators under a variety of general names such as "Stress Physiology", or "Physiological Ecology", the rise of Exo-biology and Space Biology has created intense interest in the concepts of extra-terrestrial environments and their simulation. The use of such terms as "Space Biology" or "Exo-biology" does not alter the status of this area as a legitimate part of General and Comparative Experimental Biology, and the study of Exo-biology may contribute significantly to many branches of ordinary Terrestrial Physiology, Biochemistry, Embryology, Microbiology, Genetics, and other sciences. Meanwhile, exo-biological simulation has become a powerful tool of research, calling upon many physical, chemical, and engineering specialties for support.

Simulation is a means for applying stress to the biochemical system, and has helped in the elucidation of new metabolic processes and products. More important perhaps, it is a unique tool for creating familiar and unique selection pressures. Indeed, the flowering plant has now been tested against conditions that ceased to exist long before its rise during the Cretaceous (or have never existed upon this planet). As simulation techniques become more refined and diversified, it may be possible, by combining physiological stress and genetic selection, to produce organisms with novel and "unearthly" properties which permit them to thrive in eco-systems quite different from our own, including those characteristic of Mars.

APPENDIX I

General Studies: Variations in Air and Oxygen Pressure

A. Plant and Animal Performance at the High Mountain Level - The flora and fauna of the upper biotic zone in the Himalayan range (18,000-20,000 feet $P_{atm.}$ = 1/2 sea level) are not abundant, but include flowering plants - grasses, sedges, composites; higher invertebrates - reptiles, bees, flies, spiders; and transient vertebrates - birds, rodents, carnivores.

In examining the factors of the upper Himalayan environment, total pressure and oxygen pressure have been studied independently. Experiments were done both at P_{total} = 760 mm, P_{O_2} = 76 mm (remainder N_2 or Ar), and at P_{air} = 380 mm.

It has long been known that 380 mm air pressure is far from the incipient anoxic level, even for many mammals. Symptoms of oxygen deficiency are not displayed by cats and dogs until P_{air} = 250-270 mm, and not by rabbits until P_{air} = 200 mm. The frog is not disturbed until $P_{air} \cong 100$ mm.

In our experience, Wasps (Vespa), Bees (Bombus), Ants (Monomorium), Beetles (Adalia, Passalus), other insects, and the common turtle Pseudemys can live actively for at least many weeks in 10% O_2 at sea-level pressure or in air at a simulated 20,000 feet. Neither ground locomotion nor flight is impaired, although the air density at 20,000 feet is near the lower limit for sustained wasp or bee flight.

Nematodes and protozoa in water appear unaffected by reductions in pressure to 380 mm, and the hatching rate of the brine shrimp Artemia is higher at 380 mm than at 760 mm.

The fungus Alternaria grows at twice the sea-level rate in a simulated 20,000 foot atmosphere. The germination of beet, marigold, rice, and turnip is optimal under 10% O_2 rather than air, and the germination of tomato, tobacco, and celosia seed is the same in air and 10% O_2.

Celosia, cucumber, and barley seedling growth rates are about 30-40% higher in 10% O_2 than in air; the shoot of peppermint elongates 42% faster in 10% O_2.

Optima for some species may lie at even lower partial pressures of oxygen. Thus, if barometric pressure or P_{O_2} were determining in Montane ecology, the high altitudes should be teeming with plant and animal life. In the Himalayas, the desiccating, gale-force winds, low mean year-around temperatures, and relatively brief warm season may determine the relative scarcity of organisms. Indeed, the beneficial effects of 10% O_2 at 760 mm total pressure or P_{air} 380 mm (which seem to be interchangeable) may aid organisms to withstand the otherwise lethal stresses at 20,000 feet.

B. Plant and Animal Performance at P_{O_2} = 760 mm - In contrast to beneficial effects of a moderately sub-atmospheric oxygen level on plants and its ready acceptance by animals, pure oxygen at 1 atm. comes close to being a "universal poison" (Table I-B1). It is known that Man cannot function for more than brief periods in one atmosphere of pure oxygen, and that "Prolonged exposure ... eventually produces inflammation of the lungs, respiratory disturbances, various heart symptoms, numbness of fingers and toes, and nausea."*

C. The Responses of Invertebrates to Air Pressures < 0.2 atm. (< 150 mm.) - There is a wide difference in atmospheric pressures and partial pressures of O_2 between the biotic zone of the high mountains and the surface of Mars. This gap of about one order of magnitude in total pressure and two orders in oxygen pressure presumably has been filled in many other planetary systems. In this solar system, it may be filled in the future either in orbital stations or at ground-based lunar or Martian stations and it is important that its biological effects be known.

Atmosphere tests were carried out in 4 or 16 liter glass jars closed with gasketed threaded aluminum caps equipped with gassing valves and vacuum gauge. Some tests were carried out under Plexiglas hemispheres (radius ca. 10 cm.) which could be sealed onto O-ring-gasketed bases at 1 atmosphere and then evacuated or gassed. No specialized equipment was required for the study of smaller invertebrates

* "Space Handbook: Astronautics and its Applications," pp 110-11. Staff Report, Select Committee Astronautics and Space Exploration, House Doc. No. 86, U. S. Government Printing Office, Washington, D. C. (1959).

Table I-B1

Biological Responses to 100% Oxygen at 1.0 Atmospheres

Process	Organism	Response
Seed Germination	Bean	Suppressed 90% (4 Days)
	Pea	Suppressed 100% (4 Days)
	Portulaca	Suppressed 71% (3 Days)
Shoot Growth	Pea	Suppressed 55% (length, 14 Days)
		Suppressed 62% (wt., 14 Days)
	Corn	Suppressed 51% (wt., 9 Days)
Leaf Abscission	Euphorbia	All leaves fallen (4 Days)
	Mimosa	All leaves fallen (7 Days)
Touch Sensitivity	Mimosa	Desensitized (15 hrs.)
Spore Germination	Funaria (moss)	Germ. tube 75% inhibited (3-4 wks.)
	Pteris (fern)	Germ. tube 55% inhibited (4 wks.)
Survival time	Colpidium (ciliate)	Dead 18 hrs.
	Paramecium (ciliate)	Dead 18 hrs.
	Planaria (flatworm)	Dead 14 Days (Control: 93 Days)
	Gryllus (Insecta)	Dead 24 hrs.
	Monomorium (Insecta)	Dead 48 hrs.
	Adalia (Insecta)	Dead 48 hrs.
	Bombus (Insecta)	Flight impaired 4 hrs., Dead 20 hrs.
	HeLa Cells (Man)	Cells Rounded; Dying 48 hrs.

The Leighton tissue-culture tube fitted with a rubber stopper containing gassing or evacuating tubes was useful for microscopic observation of protozoa and nematodes because it possesses flat polished surfaces.

In one series of experiments the ambient air pressure was lowered at a rate of about 0.002 atm. sec^{-1} until a behavioral change in the insects was noticed (Table I-C1). The first change was shown by the wasps and bees which ceased normal flight at about 0.4 atm., but remained highly active otherwise. All the forms studied tolerated quite low air pressures (0.1-0.17 atm.) for at least 3 days. Ten wasps maintained for 5 days at 0.16 atm. readily flew again when restored to air at 1 atm. Stag beetles and harlequin bugs were kept for 8 days and termites for 10 days at their respective tolerable pressures.

The wasp and the bumble bee can fly in 1 atm. at $P_{O_2} = 0.05$, but the former does much better at $P_{O_2} = 0.1$ atm.

Flying ability of these insects was studied in a series of atmospheres of varying density but constant (10%) oxygen content. As the atmospheric density was lowered from that of 90% Ar + 10% O_2, through less dense mixtures, down to air at 0.5 atm., a nearly 3-fold reduction, flight was unimpaired. A modest additional reduction resulted in markedly restricted flight, and at one-fifth of normal air density flight ceased completely. Further lowering of pressure caused the wasps and bees to lose coordination among walking appendages, resulting in pronounced "listing" of the body, toppling, and difficulty in righting the body once toppled. This phase was also reversible, at least within 1-2 days, when sea-level pressures were restored. Other insects were even more tolerant of low pressure. Complete immobilization generally required pressures well under 0.1 atm. In the final stages of immobilization, wasps, grasshoppers, and stag and ladybird beetles survived for 24 hrs. and recovered when a higher pressure was restored. Other insects, if kept immobilized more than two hours, displayed severe impairment in the appendages or died soon after activity was restored.

Table I-C1

General Responses of Insects to Reduced Air Pressure

Insect	Behavior at Pressure Indicated (in Atmospheres)			
	Apparently Normal, at least 3 Days	Loss of Equilibration	Complete Immobilization	Activity[3] Restored
Wasp (*Vespa*)	0.16[1]	< 0.16	0.02	0.03
Bumble Bee (*Bombus*)	0.16[1]	< 0.16	0.07	0.10
Grasshopper (*Melanoplus*)	0.10[2]	0.07	0.03	0.07
Stag Beetle (*Passalus*)	0.10	0.03	0.02	0.03
Ladybird Beetle (*Adalia*)	0.17	0.03	< 0.03	0.07
Harlequin Bug (*Murgantia*)	0.10	0.07	0.01	0.07
Termite (*Reticulotermes*)	0.10	0.05	0.03	0.05
Ant (*Monomorium*)	0.10	0.05[4]	0.03	0.07

[1] But no flight below 0.4 atm.

[2] Unimpaired jumping.

[3] After 1-15 min. at specified pressure.

[4] Only about 50% were impaired; the others moved normally.

Half of a colony of 300 Monomorium ants maintained at 0.05 atm. had impaired locomotion; however, the remainder showed a prompt, positive phototaxic response to unilateral illumination. Several trials showed the normal aerobic response time of about 5 seconds (for half of the mobile population to move to the illuminated side of the vessel) to be essentially unchanged by the 20-fold reduction in pressure.

Wasps fly normally at a total pressure of 0.5-1.0 atm., P_{O_2} = 0.1 atm., and the bumble bee was observed in flight at P_{O_2} = 0.05 atm.

Loss of flying ability in air at P = 0.4 atm. does not arise primarily from a deficiency in O_2, but to lowered atmospheric density (Table I-C2).

Locomotion and behavior of the contractile vacuole in ciliate protozoans have been examined at low oxygen pressures. Vorticella, Stentor, Paramecium, and Colpidium species were maintained in pond water at 0.013 atm. headspace pressure for three days without visible harm. The only behavioral change was that Vorticella and Stentor retained their contracted swimming form rather than their obviously stalked form.

The flatworm Planaria is rather tolerant of high O_2 concentration; nevertheless, it can live over 3 weeks in 5% O_2, and more than a day in 0.1% O_2 (Table I-C3).

The "egg" of the brine shrimp Artemia salina are notable for their shelf life of some years and ease of hatching in fresh water, seawater, or concentrated brine. We have observed that the hatching rate in 1% NaCl was higher when the head-space pressure was reduced from P_{air} = 1 atm. to P_{air} = 0.5 atm. Further reductions in pressure delayed hatching; however, about 60 out of 300 eggs still hatched to produce viable larvae under P_{air} = 0.25 atm. and ca. 20 out of 300 eggs hatched under a synthetic "Martian" atmosphere (0.1% O_2, 2.3% CO_2, 1.5% Ar, remainder N_2) at P = 0.1 atm. (Fig. I-C1). Larvae hatched at normal pressure, were readily disintegrated by decompression, but those hatched at reduced pressures remained active for periods of 4-6 days. It was of interest to note that,

Table I-C2

Atmospheric Density-Flight Relations of Vespa communis

P atm.	P_{O_2} atm.	Diluent Gas for O_2	Density gm x L^{-1} 25°C	Flying Ability
1.0	0.1	Ar	1.588	unrestricted
1.0	0.1	N_2	1.155	unrestricted
1.0	0.1	$N_2 + H_2$ 1:1	0.691	unrestricted
1.0	0.1	$N_2 + H_2$ 1:2	0.496	1-2 cm. elevation in 1 second bursts
1.0	0.1	H_2	0.203	none
0.5	0.1	Air	0.588	unrestricted
0.2	0.1	Ar	0.235	none
1.0	0.2	Air	1.177	unrestricted

Table I-C3

Lifespan of Unfed Planaria in Various Atmospheres

% Oxygen at 1 atm.	Days to Extinction (Based on 50-175 animals)
0	0.5
0.1	1.2
1	1.5
2	11
5	21
10	25
21	93
100	13

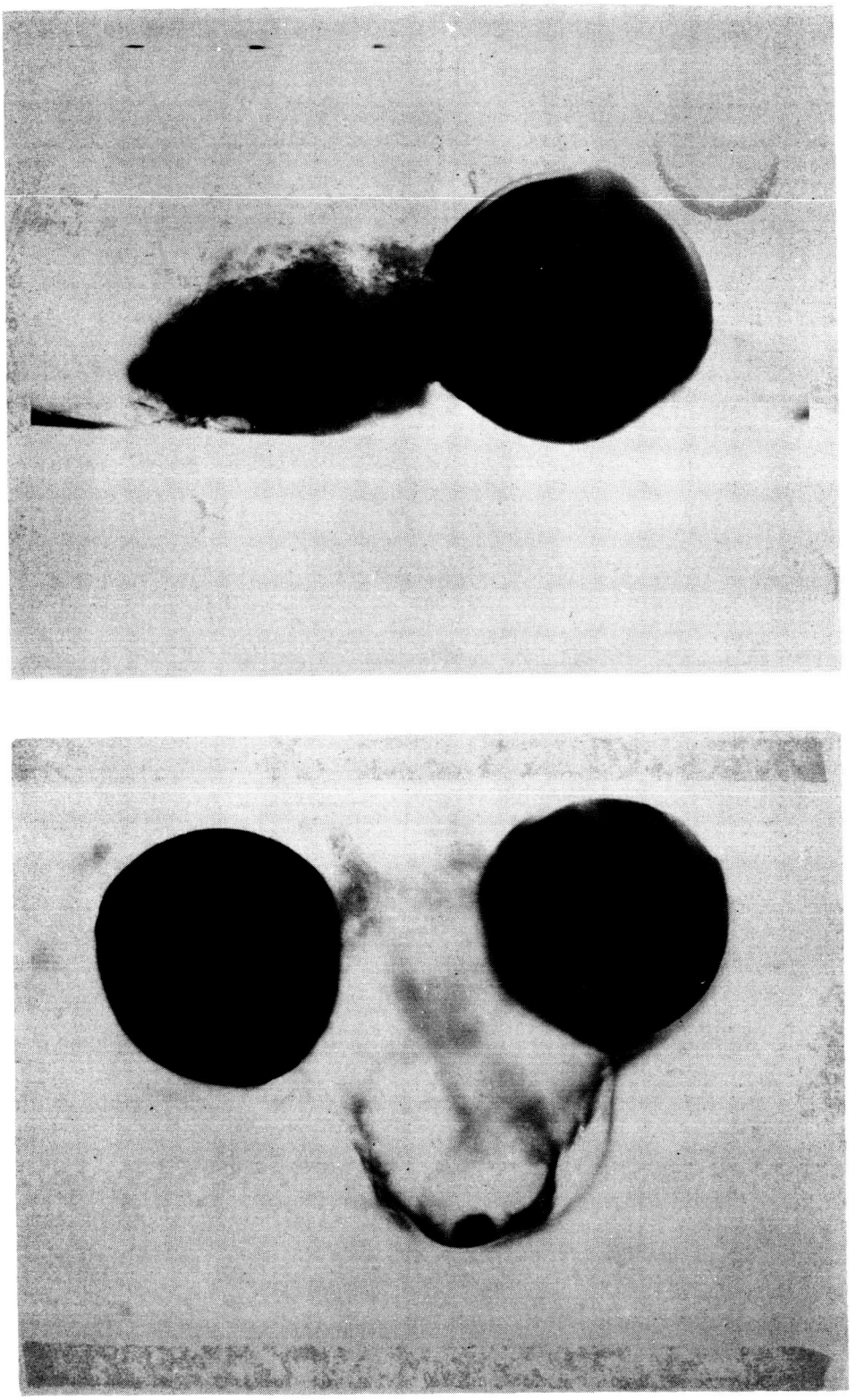


Fig. I-C1 - Hatching of the Brine Shrimp at Reduced Pressure and Oxygen Levels

Upper - Larva emerging in synthetic "Martian" atmosphere (0.1% O_2) at P = 0.1 atm.

Lower - Larva emerged in P_{air} = 0.25 atm.

Scale: Mean egg diameter = 0.16 mm.

although a few eggs hatched in 1% NaCl under the rarefied "Martian" atmosphere, none hatched when the salt level was increased to 3% or more.

Thus, even without the benefit of adaptation, selection, or acclimatization, variety of invertebrates (14 out of 15 tested) show marked capabilities under unusual atmospheric conditions. The notable exceptions were flight of insects, apparently restricted for aerodynamic rather than metabolic reasons, and survival of the planarians. In addition, mollusks have been reported by others to tolerate anaerobiosis indefinitely.

B. The Effects of Sub-atmospheric Oxygen Levels on Plants - Spores of mosses (e.g. Funaria) and ferns (e.g. Pteridium) can germinate in 5% O_2 + 95% Ar or N_2, but at one-third the rate in 10% O_2, but subsequent growth is slow.

The thallus stages of the liverwort Marchantia and the hornwort Anthoceros become discolored and shrivelled within 2 weeks at air pressures of 75-80 mm. In contrast, Marchantia has been kept in a green, unshrivelled condition for 6 weeks in 0-2% O_2 at 760 mm total pressure, but no growth was observed.

Survival tests under 0.5% O_2 (argon added to 760 mm) were run on 3-5 week-old, air-grown seedlings or cuttings. These plants were maintained in potting soil. Survival times were based upon irreversible wilting which occurs on returning the plants to air. Only minimum values were obtained in some instances, and for many species only a few plants were used. Hence, the results are highly approximate. Alyssum, Salvia, and Chrysanthemum survived for more than 24 hours, Digitaria, Oxalis and Plantago survived more than 40 hrs. The maximum survival time for Coleus was between 150 and 175 hrs., and for 10 day-old Acer, between 250 and 275 hrs. Tomato could not tolerate as much as 25 hrs. If watered with 0.005 M KNO_3, Coleus tolerated an additional 100 hrs. and Acer, an additional 25 to 40 hrs.

In general, air-grown plants can survive only for a limited time in extremely low levels of oxygen, even though the same species can be germinated and grown with little or no oxygen. Winter rye and cucumber are examples, and will be discussed separately.

Seed germination in anaerobic and microaerobic conditions will be considered later but germination capabilities of 20 species is given in Table I-D1 which shows remarkably high germination of virtually all in 5% O_2; of most in 2% O_2; and of 6 in the complete absence of oxygen.

Growth of Celosia is substantial in 5% O_2 and 2% O_2.

	Air	5% O_2	2% O_2
Rel. Whole Seedling Wt.	100	111	29
Rel. Root Length	100	115	42

Turnip shoot growth also shows striking capabilities.

	Air	5% O_2	2% O_2
Rel. Shoot Height	100	170	43

Other species showing good growth in low oxygen include peppermint, tomato, and cucumber (Figs. I-D1 and 2).

When the garden bean (Phaseolus vulgaris) is grown from seed in 4% O_2 + 95% Ar at 100% relative humidity, its appearance does not differ greatly at 2 weeks from that of air-grown plants. (Fig. I-D3a). If, however, the same plants are permitted to remain in an atmosphere of R. H. ca. 50% for 1-2 hrs., in well-watered soil, the appearance of air-grown plants is unchanged, whereas low-oxygen-grown plants collapse (Fig. I-D3b).

Speculations as to the chemical differences which could account for such novel behavior led to the discovery of constitutional and metabolic changes which are no less striking. Comparisons of these plants are summarized in Tables I-D2 and 3). Appreciable growth takes place in 5% O_2, primarily of those structures already present in the embryo.

The foregoing studies, and many others have assured us that higher plants can be grown in low oxygen atmospheres.

Table I-D1

Germination Percentage after 3-6 Days

Kind of Seed	Atmosphere: Argon (or nitrogen)	Atmosphere: 2% Oxygen + 98% argon	Atmosphere: 5% Oxygen + 95% argon	Atmosphere: Air
Lettuce	0	78	78	98
Marigold	0	33	60	57
Zinnia	0	22	60	57
Celosia	22	81	90	89
Alyssum	0	21	80	80
Portulaca	0	50	50	55
Carrot	0	50	65	32
Onion	0	65	70	69
Cucumber	17	88	96	100
Bean	0	0	60	53
Coleus	0	0	54	91
Tomato	0	2	33	91
Dianthus	7	50	50	82
Ageratum	0	30	33	84
Cabbage	0	9	71	95
Turnip	0	16	21	90
Beet	0	34	40	50
Rye	40	50	95	95
Barley	0	10	28	80
Corn	29	42	80	80
Rice	17	24	24	23

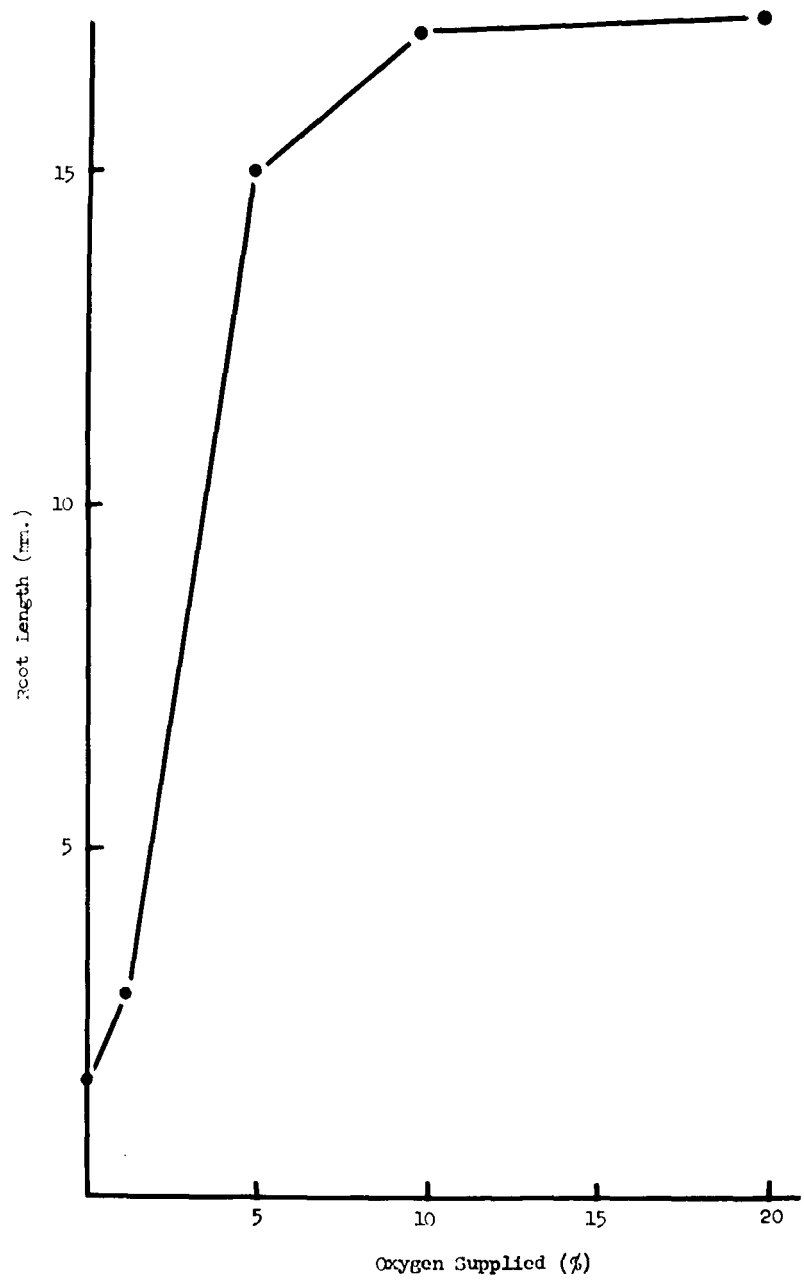


Fig. I-D1 - Oxygen-Dependency in Cucumber Root Growth

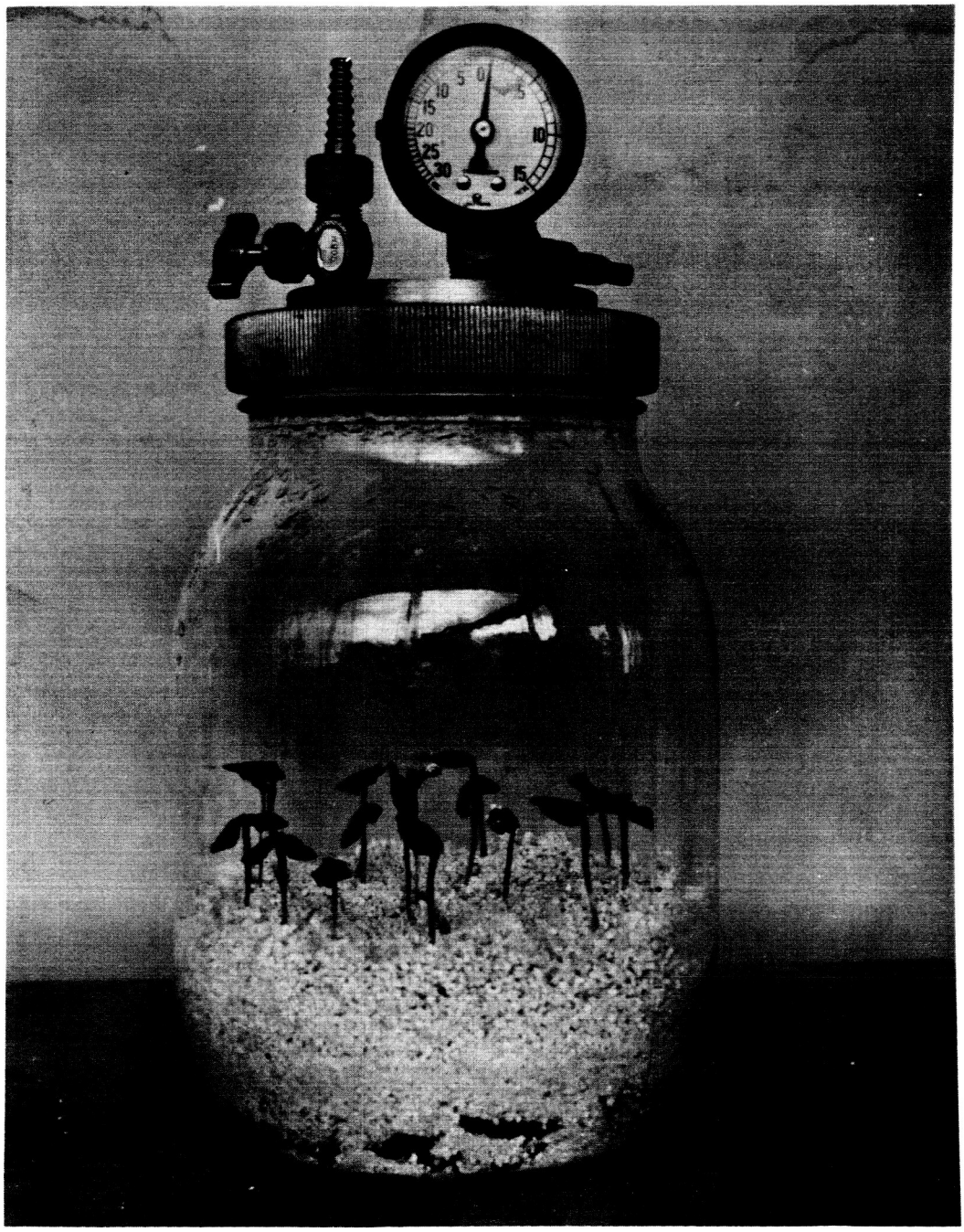
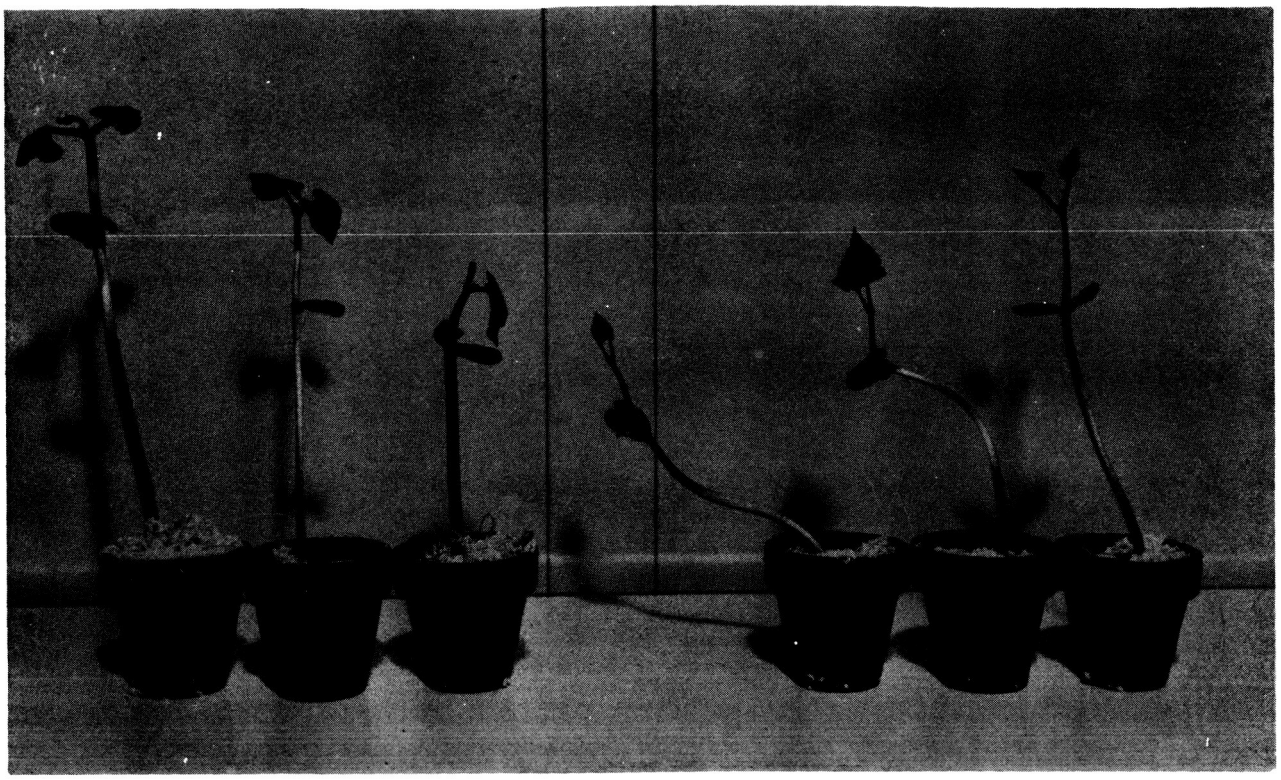


Fig. I-D2 - Sturdy Appearance of 8 Day Old Cucumber Seedlings Grown in 2% O_2



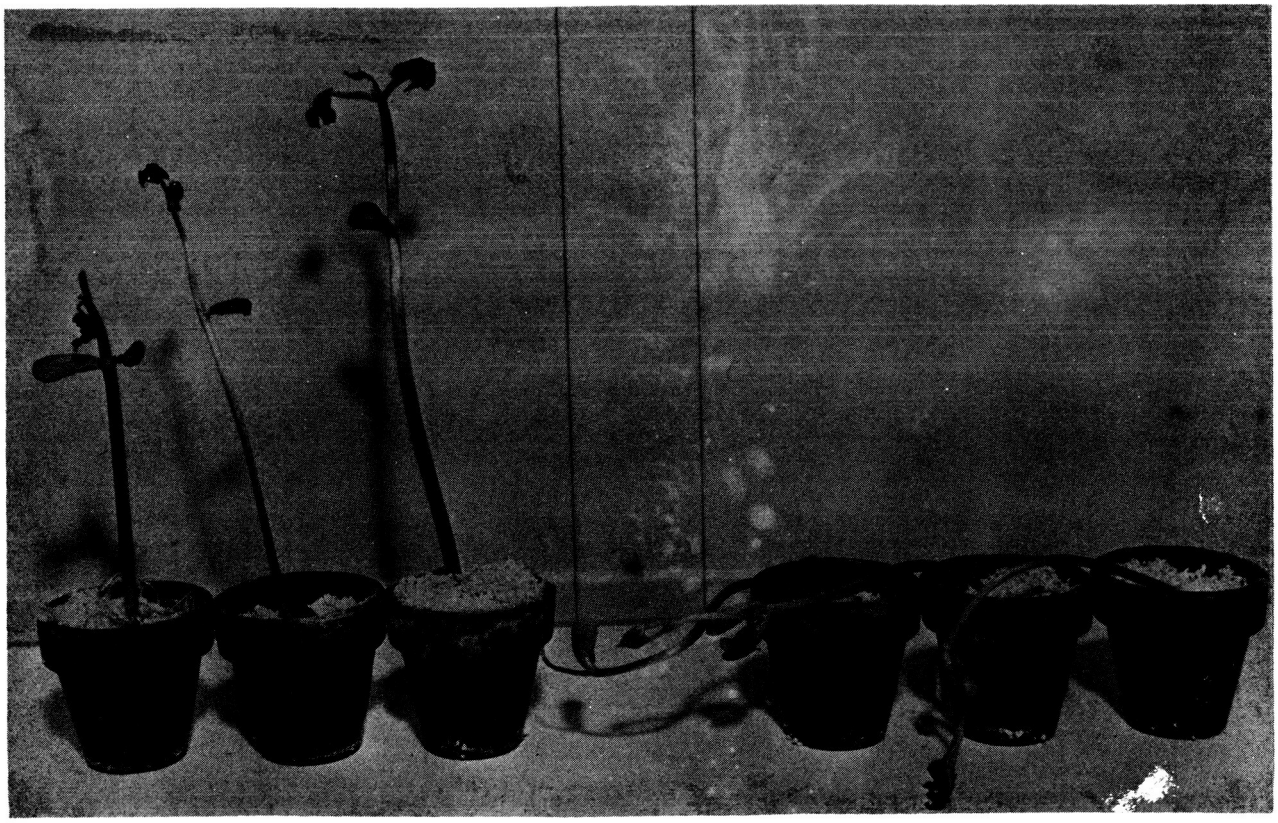


Fig. I-D3 (a) Above: Two week old bean plants immediately after removal from growth vessels. Left: 3 air grown. Right: 3 grown in 5% O_2 + 95% Ar from seed.

(b) Below: The same plants after 1-2 hrs. in Air 50%.

Table I-D2

A Comparison of Bean Plants Grown in Air and in 5% O_2:

Linear Growth Data in cm.

Organs	Measurements of Plants Grown in: a Air	b 5% O_2	b/a
Hypocotyl length	13.0	9.0	0.69
Epicotyl length			
Internode I	9.2	5.6	0.61
Additional Internodes	9.3	1.6	0.17
Total stem length	31.5	16.2	0.52
Hypocotyl diameter	0.36	0.49	1.36
Cotyledon			
Length	2.0	2.6	1.30
Width	0.46	0.85	1.85
Thickness	0.25	0.38	1.52
Primary leaf			
Petiole length	3.4	1.5	0.44
Lamina length	3.9	2.1	0.54
Lamina width	3.5	2.3	0.66

Table I-D3

A Comparison of Bean Plants Grown in Air and in 5% O_2:

Weight data in g.

Organs		Measurements of Plants Grown in: a Air	b 5% O_2	b/a
Root	Fresh	0.91	0.82	0.90
	Dry	0.05	0.04	0.80
	% Dry	5.6	5.0	--
Hypocotyl	Fresh	1.50	1.58	1.05
	Dry	0.08	0.08	1.00
	% Dry	5.4	5.1	--
Epicotyl Internode	Fresh	0.51	0.42	0.82
	Dry	0.03	0.02	0.67
	% Dry	6.0	4.8	--
Additional Internode	Fresh	0.31	0.02	0.07
	Dry	0.03	0.001	0.03
	% Dry	9.9	5.0	--
Primary Leaves	Fresh	0.40	0.10	0.25
	Dry	0.03	0.01	0.33
	% Dry	7.5	10.0	--
Cotyledons	Fresh	0.12	0.40	3.33
	Dry	0.03	0.05	1.67
	% Dry	25.0	12.5	--

Reproduction in higher plants has not yet been achieved in extremely low pressures or oxygen partial pressures, although the opening of the flower bud of the desert succulent Faucheria, and apparent beginnings of fruit growth have been observed in atmospheres containing 0.5% O_2.

Efforts to study flowering and other plant developmental processes in experimental atmospheres continue, although there are theoretical arguments that would preclude flowering at extremely low O_2 levels. Such levels must be well under 10% O_2, because that concentration is found at the biotic limits (ca. 20,000 ft) in the Himalayas, where several families of flowering plants are represented.

E. The Turtle in Experimental Atmospheres - Several preliminary studies involving oxygen pressure and other variables have been undertaken with the common red-eared turtle Pseudemys scripta-elegans.

1. Two turtles were still active after exposure to 1/2 atmospheric pressure for 31 hours.
2. Ten animals were placed in a chamber containing Ar at 1 atm. to determine anaerobic tolerance. After 12 hrs. all animals were sluggish, and by the 24th hr. all were comatose. On being returned to air all recovered within 12 to 20 hrs. Several weeks after this treatment, all animals appeared to be healthy and active.
3. Ten animals were placed in 100% O_2. No effects of this treatment were seen after 24 hrs., nor in many weeks subsequent.
4. Three turtles were placed in a large anaerobic jar lying on its side, and containing an island of mounded sand, several small rocks and water to a depth of 5 cm. Under constant evacuation and bleed in of air, the pressure was maintained at 1/2 atmosphere for 3 days. Pressure was then reduced to 0.2 atm. and held for an additional 3 days. All three turtles appeared normal. The pressure was then reduced to 0.1 atm, representing the P_{total} (but not P_{O_2}) of that of Mars. All three turtles remained active for an additional 4 days, but on

the 5th morning, with the pressure at 0.075 atm., one turtle was dead. The pressure was brought up 1 atmosphere with argon, and under a constant argon flush, the dead turtle removed. The two remaining turtles were fed for the first time in 11 days. At reduced P_{O_2} the turtles apparently need less food. Evacuation to 0.1 atm. was resumed, and the two turtles were still alive after 54 days total elapsed time.

5. Twelve animals were placed in a sand-rock-water environment in a large Plexiglas dome. Several thimbles containing food were suspended by magnets from the top of the dome.
When pressure had fallen to 0.33 atm. one animal showed distress symptoms --- possibly internal hemorrhage -- and a second was inactive. Both were removed and the decompression resumed, and was held at 0.1-0.12 atm.
On the 8th day, all animals were active and healthy. On the 9th and 12th days each, an additional death occurred. On the 12th day 500,000 units each of Penicillin and Mycostatin, and 1 gram of Streptomycin were introduced into the water and the animals were fed for the first time. An additional death occurred on the 15th day, but the remaining animals continued to be active through the 34th day. At this time, the experiment was terminated, some animals were set aside in air for observation, others were bled for hematological study.

These preliminary observations indicate that the turtle clearly is in the first rank as a candidate animal for stress and space research.

APPENDIX II

General Studies: Temperature, Water, and Other Factors

A. The Centrifuge - Although simulated high g values have pronounced effects on mammals, the results with simple organisms suggest that few highly novel or especially interesting responses will be generated. Bacterial cells, other cells, viruses, and sub-cellular particles are prepared routinely in functional states by centrifugation at more than 10^4 g.

Dry or partially imbibed rye seed were exposed to 100,000 g for periods of as long as 5 hrs. (at 0°C, in vacuo). Subsequently, germination and growth were delayed transitorily, but the seedlings soon became indistinguishable from those grown from control seed. Although sub-cellular organization may well be disturbed by such forces, the magnitude required is clearly a matter of laboratory rather than planetary interest.

Dense cultures of Paramecium exposed to a single "pulse" of 100,000 g (5 min. to maximum r.p.m. from rest and 5 min. for return to rest), were totally disintegrated. Three consecutive 50,000 g pulses had no perceptible effect upon structure or behavior. A 62,500 g pulse produced no apparent structural change, but locomotion in many individuals was impaired and erratic. In contrast, the HeLa cell, which lacks the tough wall or pellicle of Paramecium, survived 30 min. at 100,000 g; and the nematode Cephalobus, a metazoan which has about ten-fold more bulk than Paramecium, withstood centrifugation at 100,000 g for hours with no evident impairment of locomotion or activity.

The effect of more modest centrifugal acceleration on seed germination was also studied (Table II-A1). Seeds were centrifuged for 3 days in stoppered test tubes at about 30°C (heating effect in centrifuge). Controls were kept at 30°C in stoppered tubes in darkness, and also under conventional conditions in unstoppered vessels at 25°C. Centrifugation caused inhibition of germination greater than either control.

Table II-A1

Effects of Centrifugation at a Constant 100 g

During Germination (3 Days)

Species	Control A 25°C, open	Control C 30°C Stoppered	At 100 g 30°C, Stoppered
Celosia	74	82	16
Turnip	100	38	0
Coleus	87	23	0
Amaranthus	85	72	13
Carnation	95	22	9
Spinach	90	20	20
Radish	90	24	7

B. Plants (and Some Animals) at Low Temperatures - Representatives of the grasses, cacti, and crassulacae (succulents) were subjected to low temperature shock treatments ranging from exposure to -36°C to immersion in liquid nitrogen (-180°C). Exposure ranged from a few seconds to 5-10 minutes. Some results with the jade plant are summarized in the following:

Plant No.	Temperature °C	Rate of Cooling °C/sec.	Atmosphere During Thaw	New Leaves After 33 Days
8	-37	0.63	Air	10
9	-37	0.63	Argon	17
10	-55	0.77	Air	4
11	-55	0.77	Argon	12

The regeneration of leaves was thus demonstrated, and it appears that thawing in the absence of O_2 increases regenerative ability. The leaves stripped from these plants after cold treatment all rooted when planted with the cut surface in sand

Grass clumps wholly immersed in liquid nitrogen for five minutes retained their viability when thawed at 22°C, but not when subjected to accelerated warming at 40-50°C.

The succulent Sempervivum tectorum exposed for one minute to various low temperatures showed root initiation in one month as follows:

Temperature °C	Roots/5 Plants in 30 Days
Control	28
-23	12
-52	9
-80	13

Plants were hard-frozen at -10°C, and it appears that their ability to initiate roots was not further reduced by exposure to temperatures below that point.

A cactus exposed to -83°C for one minute showed damage (shrinkage and discoloration of the tip) promptly, but had produced two new branches one month after freezing.

Thus, two desert types — the dry, siliceous grass and the hydrophilic colloid-rich, thick-skinned succulent — exhibit regenerative abilities after brief exposure to extremely low temperature. In contrast, Coleus, Ageratum, and plantain were readily killed by all temperatures below -20°C.

Some seeds show an increased rate of germination following immersion in liquid nitrogen.

Species	Time of Germination Count (Hours)	Germination (%) After Immersion in Liquid N_2 for 0	1/12	1	3-4	16-19 Hrs.
Lima Bean	64	13	13	33	27	47
Winter Rye	18	0	24	36	20	80
Peanut (Jumbo Virginia)	144	5	25	10	15	35
Piñon Pine	312	4	-	28	20	28
Flax	312	8	-	20	36	52

Germination of cucumber, red kidney bean, and radish was unaffected by exposures to liquid nitrogen up to 16 hours. However, the radish seedlings grown from such seeds were 50% heavier than controls.

Germination of seeds following 24 hr.-low temperature cycling for one week was as follows (+ $\geq$ 10% germination):

	22°C 16 hrs. 4°C 8 hrs.	22°C 8 hrs. 4°C 16 hrs.	22°C 16 hrs. -30°C 8 hrs.	22°C 8 hrs. -30°C 16 hrs.
Mustard	+	+	+	-
Bachelor's button	+	-	-	-
Radish	+	+	+	-
Beets	+	-	-	-
Carrot	+	+	-	-
Cabbage	+	+	+	-
Brussels sprout	+	+	-	-
Cucumber	+	+	-	-
Chard	+	-	-	-
Turnip	+	+	-	-
Xeranthemum	+	+	+	+
Rutabaga	+	+	-	-
Bean	+	-	-	-
Rye	+	+	+	+

All the above show at least 80% germination under normal laboratory conditions. Most species cannot withstand 8 hrs. of severe freezing per day, yet a few are hardy enough to continue the germination process.

Low temperature work with animals has consisted of only a few exploratory experiments:

1. The nematode Cephalobus can be lowered to -30°C and stored for at least several days without impairment in activity when returned to ordinary temperatures.
2. Some individuals of the beetle Passalus remain active while the ambient temperature is reduced from +5°C to -20°C during 45 minutes.

After 12 hrs. at -20°C, all resume activity at +20°C.

3. Specimens of the turtle Pseudemys have survived temperatures of -5°C for 30 min.; of -2°C for 18 hrs.; and in one case continued to be active after 30 min. at -13°C.

4. The feeding medium was drained off of a 5-day-old culture of HeLa cells, and the culture flask immersed in liquid nitrogen for 18 hrs. The flask was then thawed at 25°C and 500,000 cells innoculated into fresh culture medium. After 24 hrs. incubation, the viable cell count was 64,000. Thus, some 13% of the cells survived prolonged liquid N_2 temperatures. However, when cells were allowed to contact liquid N_2 directly, they all died.

C. The Anaerobic Desert, High Altitude Salt Marsh, and Snow Dome.- Xerophytes such as Faucheria, Haworthia, Sedum, Mammilaria, Sempervivum, and Pachyphytum, have been maintained in anaerobic conditions (e.g. 100% N_2 or CO_2, or 70% N_2 + 30% CO_2) with atmospheric dew point ca. -60°C and no water save that brought in with tissues. The atmosphere was changed continuously at 7 cu. ft/hr. in chambers of about 3 cu. ft. Plants preconditioned in the N_2 + CO_2 mixture for 4 days prior to being placed in 100% CO_2 were healthy and undesiccated after 3 weeks. Those in either N_2 or CO_2 were generally shrivelled, and under CO_2, the normal green color gave way to a pale watery straw coloration. Plants under N_2 were more normal in appearance, but their weight loss (> 50%) were higher than those under CO_2.

In general, irrespective of appearance, plants anaerobically conditioned for 3-4 weeks could not survive a return to air. Within 24 hrs., the greenish plant body became greyish with streaks or spots of pink, red, brown and black pigmentation, and became desiccated in spite of unlimited water. The "Tiger Jaw" Faucheria opened a flower bud in one instance, and the beginnings of fruit development were observed.

Rutabaga and turnip roots will grow shoots readily if left in the laboratory atmosphere without supplementary water. In the chamber conditions described above, these roots failed to support existing shoots, or to grow new ones; and the rate of shrinkage of the roots was higher than for other plants tested.

Coleus, which is easily wilted in air if not watered, was severely desiccated when in the chamber for a few hours. If, however, one intact branch is enclosed in a polyethylene bag containing water, the remaining exposed portion of the plant can survive 1-2 weeks of desert chamber. A defoliated stem cannot carry out the required absorptive functions (Fig. II-C1).

The coats of many seeds are coated with extremely hydrophilic polysaccharide mucilages which begin to hydrate and swell rapidly when the seed encounters water. Range and semidesert grasses such as "alkali sacaton" (Sporobolus spp) and open dryland forms such as flax, wild mustard, plantain, etc. are common examples of seeds with water-holding coats. Conceivably, this is the sort of adaptation one would expect in any area which had moved slowly from well-watered condition to desert — a possible situation on Mars.

Many common seeds native to the moister regions lack such an adaptive device but it can be conveyed upon them in an approximate way by embedding them in 2% agar gel. Disks of the seed-containing agar were placed in the "desert" chamber on dry Perlite under N_2. Thirteen species have been tested in 5-day trails. Sunflower, onion, and nasturtium failed to germinate; chinese cabbage, carnation, cockscomb, and okra yielded up to 10% germination; morning glory, spinach, and cucumber gave up to 30% germination; and hibiscus and Winter rye, over 50% germination.

Other observations made in the desert chamber include:

a. After 6 weeks in N_2, the common xerophytes were dead and so

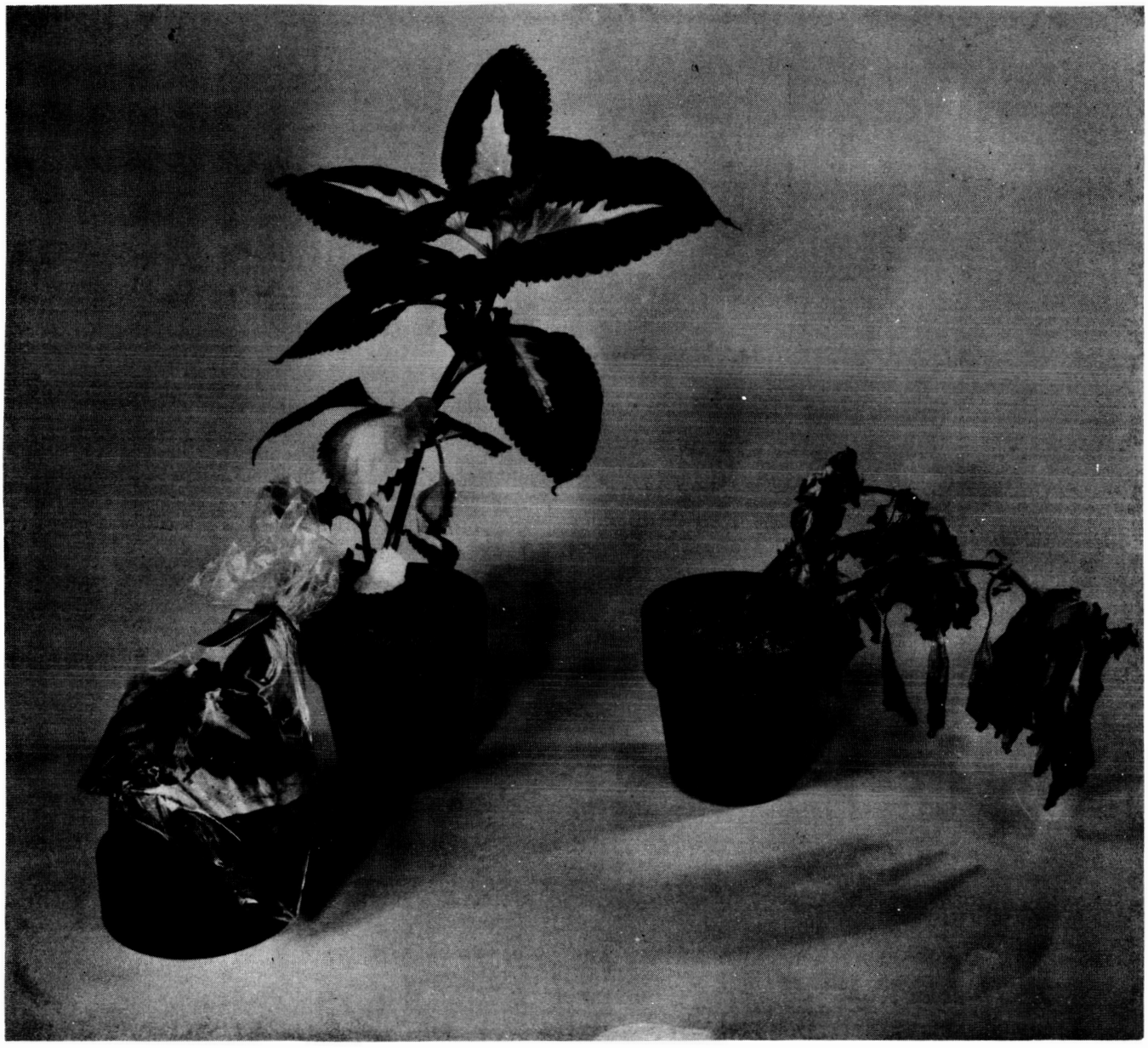


Fig. II-C1 - Effect of Feeding Water to Coleus Through a Leafy Branch on Desiccation Resistance.

desiccated that they crumbled when touched; however, they exhibited superficial patches of a filamentous material. Several of these patches were identified as the phycomycete Mucor mucedo complete with sporangiophores and sporangia (Fig. II-C2). Other fungi include a dense white imperfect form as yet unidentified, and another form which may be Botrytis.

b. Passalus, the Texas Stag Beetle, and Pseudemys, the turtle, can withstand the N_2-desert for 24 hours without mortality. They are active for about half of this period, then become rapidly sluggish and comatose, but can be revived in air.

The "High Altitude Salt Marsh", offers another means for examining a dual stress condition involving water and atmosphere which appears to offer as much promise as the alternative desert condition. The salt marsh consists of a moderately concentrated solution of NaCl or NaCl + Ca $(NO_3)_2$ covering a perlite substratum to a depth 1-2 cm contained in a vessel evacuated to P_{air} = 0.1 atm. Fluorescent tubes of about 400 foot-candles supplied illumination. In a mixed salt solution totalling 5,500 p.p.m., rye began to germinate after 18 hrs., carnation and lima bean after 48 hrs., and Celosia after 4 days. Borage did not germinate until the 8th day, okra after 2 weeks, and Ageratum failed completely. At the end of two weeks, four species out of 7 had passed the emergence phase, and the growth of the plants was as follows:

	Height, cm	No. leaves/plant
Rye	25	2
Carnation	roots only	-
Lima Bean	15	4
Celosia	3	2
Borage	4	2
Okra	roots only	-
Ageratum	failed	-

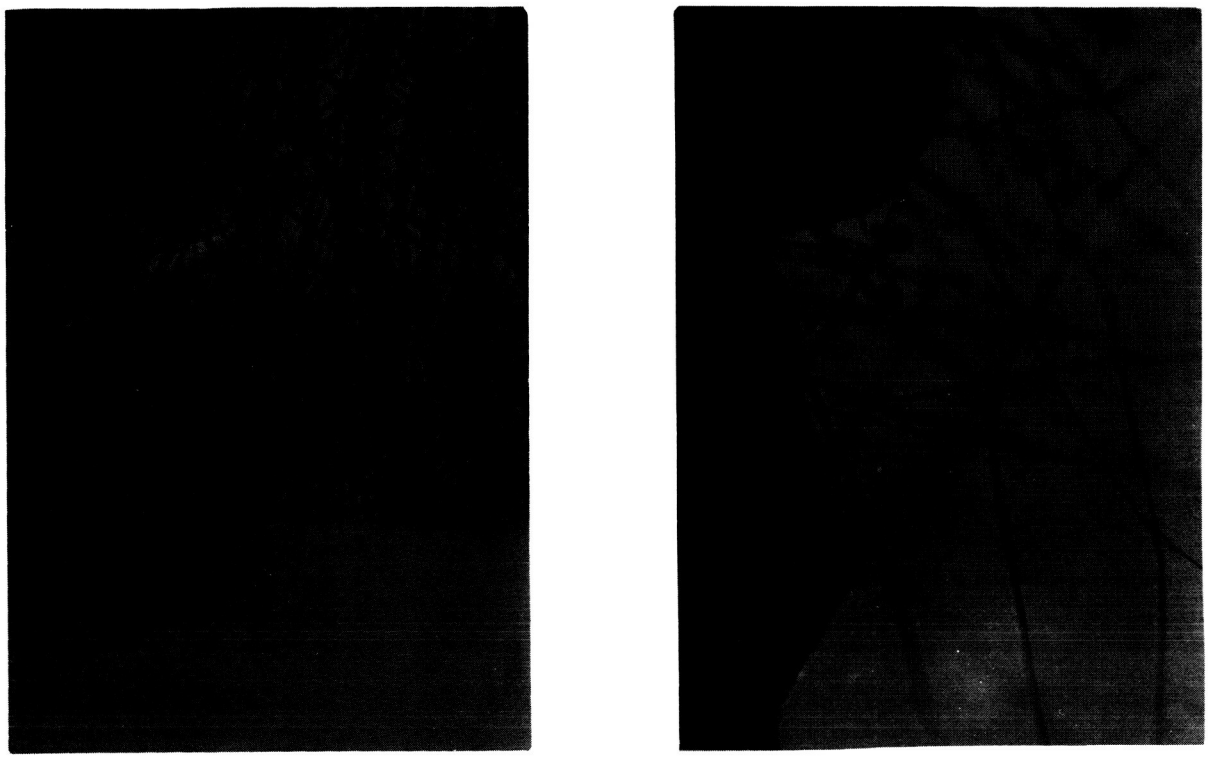


Fig. II-C2 - The Mold Mucor growing and sporulating on the dead surface of a xerophyte in the desert chamber.

In NaCl alone at 12,000 p.p.m. rye germination began in 2 days, and reached 20% in 8 days. At this time shoots were 0.1 cm. high. In NaCl + $Ca(NO_3)_2$ at 24,000 p.p.m., 100% germination occurred in 8 days, the seedlings being 0.2 cm tall. In NaCl + $Ca(NO_3)_2$ at 24,000 p.p.m. in air, germination at normal pressure was also 100%, but 5 cm seedlings had grown. Lima bean could not germinate at all in these higher salt levels.

The "Snow Dome" has been used to study seed germination at P_{air} = 0.1 atm. and low water supply. A shallow dish containing 5 ml. of water is placed in the dome. In one type of experiment water simply evaporates into the atmosphere - about 3 ml. being transported in 5-6 days. Alternatively, water vapor in the atmosphere is condensed onto liquid N_2 cooling coils and the snow is dislodged by a vibrator onto seeds planted below. By condensing snow for one hour in each 24, all the water is deposited as a layer 0.033 g/cm^2 during the five-day period.

Germination percentages with and without freezing are:

	Water as	
Species	Vapor	"snow"
Cucumber	0	0
Carnation	0.4	10
Celosia	10	16
Rye	27	56

D. Atmosphere-Conditioned Cold Resistance in Cucumbers and Beetles - The interaction of low temperature and low oxygen pressure as environmental factors is illustrated by the difference in cold resistance of cucumber seedlings grown in air as compared to those grown in 2% O_2 + 98% Ar (Fig. II-D1a and b).

Seedlings were lowered to -10°C, held there for 1 hr., and then

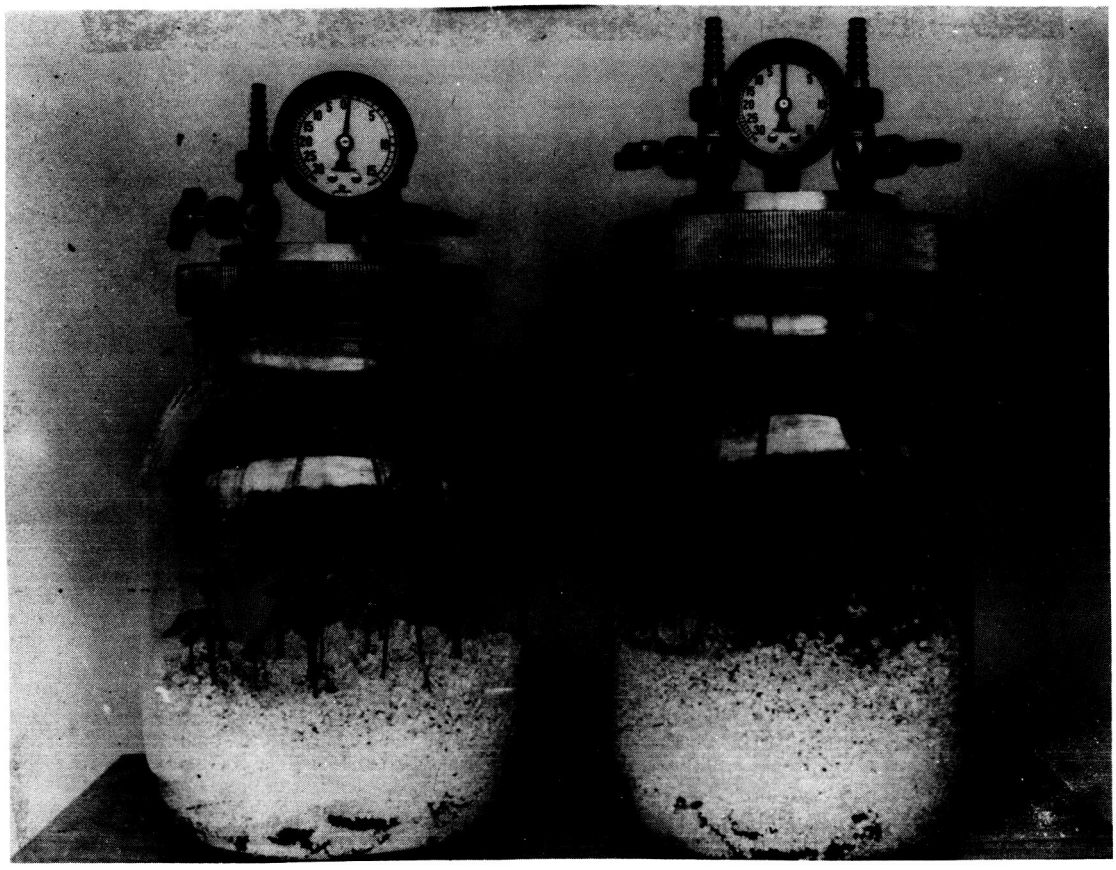


Fig. II-D1 (a) Freeze-resistance of cucumber seedlings grown in 2% O_2 - 98% Argon (right).

(b) Freeze-resistance of cucumber seedlings grown in 2% O_2 - 98% Argon vs. Air (left).

allowed to thaw at room temperature. Every air-grown seedling suffered mechanical damage and was collapsing, yet not one of the low-oxygen plants displayed any sign of injury at the time of thawing or several weeks later.

The temperature-atmosphere interaction can also occur during germination, as shown by Japanese turnip, cabbage and Kale at 6°C.

	% Germination	
Species	98% Argon 2% O_2	Air
Turnip	33	0
Kale	48	7
Cabbage	31	0

In contrast, anaerobic conditions do not increase the relative germination of rye, onion, or barley at low temperature.

Air pressure has a profound influence upon the effects of cooling the beetle Passalus from +25°C to -7°C. Behavior during cooling, persistence of activity at -7°C, and recovery were all markedly better at reduced air pressures, as compared to air at normal pressure.

Air Pressure Atmospheres	Responses During Cooling Period	Activity at -7°C	Recovery - at 72 hrs.
1.0	convulsions	none	2 out of 3 dead, 1 normal
0.5	slowed gradually	none	3 out of 3 normal
0.1	slowed gradually	1 out of 4 still moving	4 out of 4 normal

E. Effect of Substratum on Growth - A comparison between perlite and hematite (each supplied with inorganic nutrients) as growth media at 1 atm. and 0.1 atm. of air has been carried out with rye and cucumber. The experiments

were short-term — 8 days — but significant differences were noted.

Winter Rye

Measurement	Perlite	Hematite	H/P
	Air - 1.0 atm.		
Shoot length, cm	8.2	12.5	1.53
Root length, cm	9.0	6.0	0.67
Fresh wt., gm/seedling	0.13	0.33	2.54
	Air - 0.1 atm.		
Shoot length, cm	0.7	12.3	17.6
Root length, cm	0.9	9.7	10.8
Fresh wt., gm/seedling	0.06	0.40	6.7

Cucumber

Measurement	Perlite	Hematite	H/P
	Air - 1.0 atm.		
Shoot length, cm	1.2	1.6	1.33
Root length, cm	4.6	4.0	0.87
Fresh wt., gm/seedling	0.09	0.19	2.11
	Air - 0.1 atm.		
Shoot length, cm	0.7	1.1	1.56
Root length, cm	1.1	5.3	4.82
Fresh wt., gm/seedling	0.19	0.40	2.11

In both species, it is evident that hematite has a similar pattern of effects upon ordinary aerobic growth, but its stimulatory effect upon the rye seedling and upon cucumber roots at low pressure is truly striking. Plants were well supplied with nutrients both exogenous and endogenous including

soluble iron. Many experiments have shown that Fe salts do not stimulate growth at reduced O_2 levels. Thus, the cause of stimulation resides in other properties of the growth medium.

The stimulatory effect of elemental sulfur upon germination was even more surprising than the effects of hematite. Sulfur was combined with about one-tenth its volume of silica gel and "whipped" with slow addition of water until it had the consistency of a stiff paste. Seeds were placed upon the sulfur surface just as they are routinely on moist filter paper. Under 2% O_2 + 98% Ar, the effect of sulfur was:

	Germination (% at 5 Days)	
	On filter paper	On sulfur
Lettuce	8	24
Turnip	16	24
Cucumber	50	81

Under 5% O_2 + 95% Ar, its effect after 2 days was:

Lettuce	61	83
Turnip	21	43
Cucumber	16	64

Anaerobes germinated in argon were affected by sulfur, but seeds germinated in 100% O_2 were not. When sulfur is melted and cast in dishes, whether melting took place under air or argon, the stimulatory effect of powdered sulfur disappears.

Root Length of Cucumber (mm)

		On paper	On sulfur
In Argon	5 Days	2.0	6.1
In Air	1 Day	12.3	14.2

In an unsuccessful attempt to explain these wholly mystifying effects with sulfur, a number of compounds from Periodic Group VI were tested with cucumber and rye under 5% O_2 and air. These included $S^=$, $SO_3^=$, $SO_4^=$, $Se^=$, $Se_2^=$, $SeO_3^=$, $SeO_4^=$, and $TeO_3^=$. Only tellurite at 0.01-0.02 M concentrations increased germination in cucumber and rye, but inhibited root and shoot growth moderately, but, surprisingly, none inhibited seedling growth markedly. To illustrate:

a. Cucumber in 5% O_2 in water yielded 15 mm roots in 3 days; in 0.01 M K_2TeO_3, 12-mm roots had grown, and at 0.02 M, roots were 9-mm long. Germination rates were somewhat elevated with tellurite.

b. Rye in 5% O_2 in water yielded 46 mgm seedlings at 3 days; in 0.02 M K_2SeO_3, 33 mgm seedlings were produced, and in 0.01 M Selenite seedlings were 42 mgm in fresh wt.

Similar results were obtained with selenate, selenide, and diselenide.

F. <u>High Temperature and Radiation - Some Preliminary Notes</u> - Seeds are noted for longevity and heat resistance. They can be exposed to temperatures of 75-100°C — and sometimes higher — for minutes to hours without losing viability. The more resistant types include some of the grasses and especially oil-rich seeds such as the mustards and flax. Heat damage increases with increasing oxygen pressure and water content of the seed.

O_2(%) During Heating	Growth of Bean (% Unheated Control) After Exposure to 105°C for 60 min.	Water Content of seed (%)	Germination of Crabgrass (%) After Exposure to 75°C for 30 min.
21	10	9	50
10	10	27	29
0.005	42	34	27
		40	1

Preheating of seed can also condition subsequent responses to light:

Seed	Normal Effect of Light	Treatment which changes Light Effect
Crabgrass	Nil	75°C 60 min ⟶ Absolute Light requirement
Radish	Nil	100°C 60 min ⟶ Severe Photo inhibition
Dandelion	Slight Stimulation	100°C 60 min ⟶ Photo-inhibition

Brief exposures to heat — for example 50-60°C for periods of 15-30 min. can actually stimulate seed germination, an effect which has been seen in corn, rice, and cucumber.

Some seeds can also tolerate combined low and high temperature shock such as liquid N_2 to 100°C with good viability and vigor.

These are only token observations to indicate that high temperature as a shock or conditioning factor merits study. What is true for the seed is also commonly true for spores of other organisms. In any broad environmental picture it is necessary to take into account the ability of life to withstand extremes of environmental variation when in a state of dormancy.

The aerial organs of the begonia plant were discolored and dying after a dose of ca. 5×10^8 ergs/cm^2 of 2537 Å radiation, commonly recognized as mutagenic and sterilizing. This dosage of ultraviolet had no immediate effect on sansevaria, but after nearly 2 weeks, some delayed injury was evident. Ivy was not extensively affected by a somewhat higher ultraviolet dosage. The annual u.v. above 3000 Å on Earth and Mars is about 10^{10}-10^{11} ergs/cm^2.

Turtles exposed to ca. 3×10^8 ergs/cm^2 of 2537 Å even when in 100% O_2 (1 atm.) were unharmed. The animals did not try to avoid the radiation,

but rested with their necks fully stretched and eyes directed toward the source.

With respect to ionizing radiation, relatively large dosages are required to prevent germination and seedling growth, as the following examples show:

Dose Kr (250 KVP)	Growth of Flax One Month after Seed Irradiation (control = 100)		
	Root	Hypocotyl	Epicotyl
0	100	100	100
2.3	106	85	98
10.7	115	84	98
19.1	113	85	113
44.3	85	49	55
76.4	76	39	13

Dose Kr (250 KVP)	Growth of Bean Embryos 3 Days after Irradiation (control = 100)		
	Main Axis	Root Hairs	Branch Roots
0	100	100	100
20	92	80	40
40	92	85	0
60	91	75	0
75	91	55	0
150	91	0	0

APPENDIX III

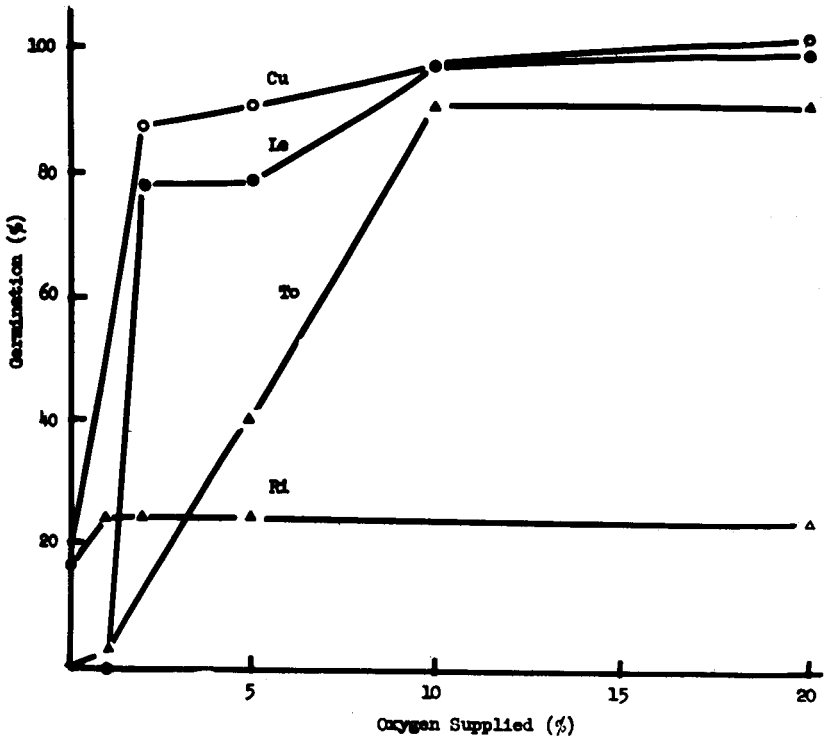
Biochemical and Physiological Observations on Organisms in Simulated Environments

It is impossible at present to attempt an analysis of the biochemistry of non-terrestrially-conditioned earth organisms. The biosphere as it is now constituted contains organisms equipped to cope with stress factors such as anaerobiosis and desiccation. The anaerobic bacteria and other microorganisms, can carry out all of the essential processes involved in synthesis of nucleotides, nucleic acids and proteins.

It is somewhat surprising, therefore, that biochemists still emphasize mechanisms of aerobic metabolism and energetics as if they were the essential mode of existence, when this is clearly untrue. The highest orders of animal life on this planet require O_2, but this should not mislead us into projecting too literally the need for highly specific earthly elements of life support onto other planetary systems. This becomes especially evident in view of what has been demonstrated, namely that environmental factors and systems, quite different from present terrestrial conditions, are suitable for terrestrial forms, even without the benefit of genetic selection.

A. Germination - The response of seed germination to variations in P_{O_2} is highly variable according to species (Fig. III-Ala). Lettuce shows an almost all-or-none response, being incapable of germinating in 1% O_2, but almost fully capable in 2% O_2. Cucumber also shows a steep response curve, which begins with a clearly anaerobic component onto which the introduction of O_2 adds the activation of members which are obligate aerobes. The requirements of a tomato seed population are spread over a considerable range of O_2 concentrations, whereas rice is essentially "indifferent"

The major product which distinguishes aerobic metabolism from fermentation is ATP, erroneously designated "high-energy phosphate", which provides, in the free energy of the pyrophosphate bond, the driving force for many biosynthetic reactions and biochemical work. Can ATP replace O_2 in anaerobic conditions? The answer is equivocal in most situations because ATP supplied externally may be hydrolyzed



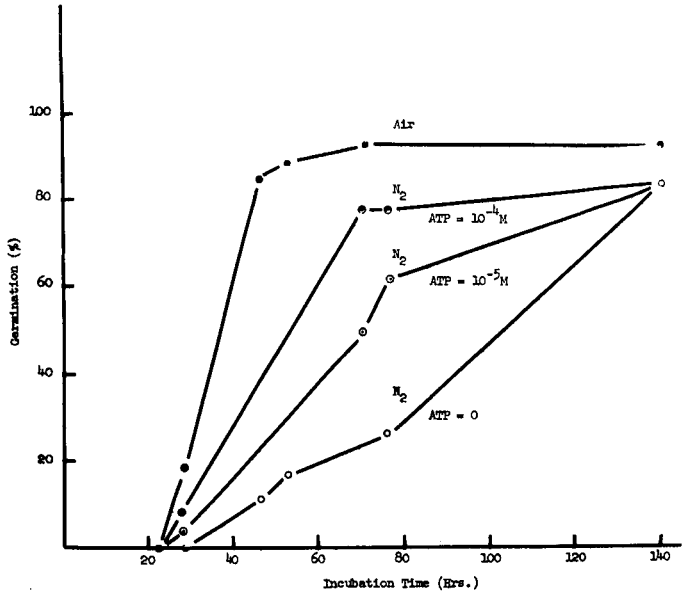


Fig. III-A1 (a) (above) The Dependency of Germination Rate Upon P_{O_2}. Species include rice (Ri); tomato (To); lettuce (Le); and cucumber (Cu).

(b) (below) Effect of ATP Upon the Germination of Celosia under Anaerobic Conditions.

enroute to its target. Nevertheless, a clear affirmative answer has been obtained in a few instances, most strikingly in the case of the germination of Celosia (Fig. III-A1b) . This species is a capable facultative anaerobe, but responds quantitatively to O_2. In the presence of ATP the slow anaerobic curve approaches the steep aerobic curve for germination vs. time.

In addition to the elimination of the need for O_2 by supply of its metabolically produced equivalent, the use of alternate electron acceptors has been considered. These include Fe (III), Ce (IV), $Fe(CN)_6^{\equiv}$, and NO_3^-. Only the last of these has given definitive results as shown in this example with Winter rye:

KNO_3, moles/L	Germination, %
0	60
0.001	78
0.005	99
0.01	89
0.05	88

On the other hand, rye gives evidence of possessing a completely different metabolic system, known as the Stickland reaction and found in the obligate anaerobic genus Clostridium.

$$H_2NCH_2COOH + ADP + H_2PO_4^- + R(SH)_2 \longrightarrow CH_3COOH + ATP + NH_3 + RS_2$$

When rye was supplied the components of this system singly and in combination under anaerobic conditions, the results at 7 days were:

Substance	Germination, %	Shoot Length, mm
K_2HPO_4, 0.01 M	35	1.0
+ Glycine, 0.01 M	45	2.9
+ ADP, 0.01 M	60	2.1
+ ADP, Glycine	80	3.1
ADP + Glycine, without phosphate	40	2.5

It was assumed that under anaerobic conditions thiols need not be added. Under low oxygen conditions, the reducing power of seedlings builds up appreciably, and

therefore, the $R(SH)_2$ should be continuously regenerated.

Carbon monoxide is among the unusual metabolic products of seeds germinating in low oxygen or anaerobic conditions. Approximately 50 cm^3 of loosely packed seeds and an equal volume of oxygen-free water were introduced into 500 cm^3 polyethylene vessels which were evacuated, filled with 5 percent oxygen plus 95 percent argon, and held at 25°C. After 2 days, Winter rye, Alaska peas, Marketer cucumber, Purple Top White Globe turnip, and Black Seeded Simpson lettuce had formed traces of CO, whereas Golden Bantam corn, Red Kidney bean, and Marglobe tomato had not. The same results were obtained whether or not the seeds were sterilized in 0.5 percent NaOCl. After 5 days, appreciable quantities of CO were present in the atmospheres of several species (Table III-A1). Schiff tests showed that volatile aldehydes are generated by some species and not by others, but the results were too variable to suggest a relationship between aldehydes and CO.

Infrared spectroscopy and gas chromatography indicate that rye germinated in 5% O_2 or less, generate small quantities of butyric acid and C_4 or C_5 alcohol.

Rye, cucumber, soybean, and other seeds also produce hydrogen during germination at sub-atmospheric O_2 levels. It has not been proven that the highly sterilization-resistant bacteria within the integuments of the seed are not responsible for this phenomenon. In any case, there is reason to suspect that there is a mutualistic feature to H_2 production in seeds, involving the dependence of the bacteria upon seed metabolites, the relation between growth and H_2 formation, the beneficial effects upon germination of lowered P_{O_2}, and the direct stimulation of seed germination by H_2. Thus, low oxygen environment may bring forth new symbiotic relationships in recognizable form.

B. Bean Plants Grown in 5% O_2 + 95% Ar - Some growth and morphological characteristics of beans grown in 5% O_2 nave been given in Appendix II. Tables III-B1 and III-B4 show the essentials of elementary analyses, constitutional changes during seedling growth, amounts of selected enzymes and other constituents, and the differential carbon disposition on an atom of carbon basis.

Table III-A1

Carbon Monoxide Production by Seeds After 5 Days at Reduced Oxygen Levels. Schiff Test for Aldehydes: N, Negative; P, Positive; PP, Intense Positive; W, Weak

Seed and wt. of seed used (g)		CO level ppm	CO level µg/g seed	Schiff test
Rye	(39)	25	1.95	N
Maize	(44)	0		W
Pea	(44)	10	0.36	P
Bean	(42)	0		W
Tomato	(14)	0		N
Cucumber	(34)	15	1.04	P
Turnip	(35)	10	0.69	N
Lettuce	(24)	10	0.48	PP

Table III-B1

Elementary Composition and Weight Data for Bean Seedlings Grown in Air or Sub-Atmospheric Oxygen Levels

Constituent	Seed	Seedlings Grown 2 Weeks in					
		Air			5% O_2 + Air		
		Root	Shoot	Cotyl.	Root	Shoot	Cotyl.
C	42.3	41.5	42.2	41.4	43.1	42.6	41.9
H	6.3	6.2	6.6	6.3	6.2	6.5	6.6
O	38.9	34.4	37.8	37.0	35.1	36.3	37.4
N	4.5	6.6	6.6	4.2	6.6	6.4	5.4
S	0.4	0.8	0.6	0.8	0.6	0.8	0.5
Ash	6.1	8.5	6.0	9.2	7.7	5.2	7.8
Total Dry Matter, mg.	530	50	200	60	40	120	100
Water, mg.	24	860	2920	180	780	2100	700
Total Plant Wt., mg.	554	910	3120	240	820	2220	800

Table III-B2

Changes in Elementary Constituents of Bean Seedlings
Cultivated in Air and 5% O_2 + 95% Argon

Stage	Constituent (mgm/plant or plant part)					
	C	H	O	N	S	Ash
Seed	224.2	33.4	206.2	23.9	2.1	32.3
2 Week Air-Grown Seedling						
Root	20.8	3.1	17.2	3.3	0.4	4.3
Shoot	84.4	13.2	75.6	13.2	1.2	12.0
Cotyl.	24.8	3.8	22.2	2.5	0.5	5.6
Total	129.0	20.1	115.0	19.0	2.1	21.9
$\Delta_{Total-Seed}$	- 95.2	-13.3	- 91.2	- 4.9	0	-10.4
2 Week Low-Oxygen Seedling						
Root	17.2	2.5	14.0	2.6	0.3	3.1
Shoot	51.1	7.8	43.6	7.7	1.1	6.2
Cotyl.	41.9	6.6	37.4	5.4	0.5	7.8
Total	110.2	16.9	95.0	15.7	1.9	17.1
$\Delta_{Total-Seed}$	-114.0	-16.5	-111.2	- 8.2	-0.2	-15.2
$\frac{\Delta \text{ 5\% } O_2}{\Delta \text{ Air-Grown}}$	1.19	1.24	1.22	1.67	-	1.46

Table III-B3

A Comparison of Bean Plants Grown in Air and in 5% O_2: Chemical Composition (as % dry weight), Selected Enzyme Activities, and Spectrophotometric Data

a. Chemical Constituents

Constituent	Plants Grown In: a Air	b 5% O_2	b/a
Chloroform extractables			
Root	8.7	9.1	1.04
Stem	15.8	7.3	0.46
Cotyledon	5.7	"0"	0
Glucose			
Root	0.44	1.06	2.41
Stem	0.34	0.63	1.85
Cotyledon	"0"	0.41	-
Sucrose			
Root	1.86	6.61	3.56
Stem	1.08	2.95	2.74
Cotyledon	3.95	5.47	1.39
Lignin			
Stem	5.8	2.4	0.41

Table III-B3

b. Enzymes

Enzyme and Organ		Plants Grown In: a Air	b 5% O_2	b/a
Peroxidase (μ moles pyrogallol oxidized/min.)				
Root	per gm. dry wt.	172	203	1.18
	per plant	8.6	8.2	0.95
Epicotyl	per gm. dry wt.	375	2,183	5.82
	per plant	23.5	43.7	1.86
Catalase (μ moles H_2O_2 decomposed/min.)				
Root	per gm. dry wt.	300	742	2.47
	per plant	15.0	29.7	1.98
Epicotyl	per gm. dry wt.	3,450	3,000	0.87
	per plant	208	60	0.29
Lipoxidase (Δ absorbancy 232.5 mμ/min.)				
Root	per gm. dry wt.	28.5	20.0	0.70
	per plant	1.5	0.8	0.53
Epicotyl	per gm. dry wt.	19.2	40.0	2.08
	per plant	1.2	0.9	0.75

c. Spectrophotometry of Extracts

Object of Measurement		Plants Grown In: a Air	b 5% O_2
Acetone leaf extract (30 ml/gm. fresh leaf)	λmax	430, 662	430, 662
	Abs. at λmax	0.847, 0.420	0.692, 0.393
Chloroform stem extract (5 ml/gm. fresh stem	λmax	238^{sh} 278, 320^{sh}	238, 272, 320^{sh}
	Abs. at λmax	0.527, 0.351, 0.110	0.855, 0.493, 0.152
Ethanol-HCl stem extract (150 ml/gm. fresh stem	λmax	272, 315	278
	Abs. at λmax	0.277, 0.129	0.665

Table III-B4

Representative Carbon Distribution in
Representative Compounds in Bean Plants
Grown in Air and 5% O_2 + Ar

Compound	Carbon in Specified Compound (atoms/1000 atms. total C)	
	Air Grown Plants	Low Oxygen Plants
Glucose (total)*	8.4	20.1
Fructose	5.2	14.1
Lignin	90.5	37.5
Methoxyl	14.0	11.5

* Free reducing sugar plus that combined in sucrose

Beans grown in low oxygen and in air contain similar percentages of C, H, and O, but low-oxygen seedlings contain a higher percentage of protein N. On an absolute weight basis, beans grown in low oxygen lose more C, H, O, N, and Ash than those grown in air. Loss of N as NH_3 was detected, and a lowered metabolic efficiency is suggested.

Data on glucose, fructose, lignin, and methoxy carbon contents recalculated on a total C basis show that beans grown in low O_2 contain more C as sugars and less C as lignin and methoxyl. An altered cell wall chemistry is suggested.

The low-oxygen plant is higher in general reducing power, higher in sucrose and glucose content, and lower in chloroform solubles and lignin. The low-oxygen epicotyl contains far more peroxidase, slightly less catalase and about twice the lipoxidase found in air-grown epicotyls. The low-oxygen root differs little in peroxidase, but contains appreciably more catalase and somewhat less lipoxidase than air-grown roots. Various tissue extracts from plants grown in air or 5% O_2 were compared with respect to absorption spectra. The measurements taken suggest that the leaf pigments are not greatly different, whereas the low-oxygen stems appear richer in the ultraviolet regions suggesting olefins, phenols, and phenols with side-chain conjugation.

C. Cucumber Seedlings in Various Atmospheres - Cucumber germination and growth have been discussed in various places in III and IV. Unlike bean, which is strictly aerobic, cucumber seed populations contain both aerobes and anaerobes. Cucumber anaerobes are relatively sluggish, but seedlings grow exceedingly well in 1-5% O_2. Elementary changes in the cucumber are shown in Table III-C1 and Fig. III-C1.

On a percentage basis, cucumbers grown in air and in low O_2 show marked differences after only 4 days. Tissue oxygen varies directly with atmospheric O_2. Two week-old seedlings grown in 5% O_2 + Ar are more juvenile as judged by chemical composition than their air-grown counterparts.

Losses in C, H, O, and N were greater in air-grown than in low-oxygen atmospheres. Loss of N as NH_3 was detected in the air-grown group. An exceptionally

Table III-C1

Elementary Composition of Cucumber Seeds and Seedlings Grown in Various Atmospheres

Constituent	Seed	Seedlings Grown 4-Days		Seedlings Grown 2 Weeks		
		Air	5% O_2 + Ar	Air	5% O_2 + Ar	1% O_2 + Ar
C	57.1	50.6	55.2	40.5	44.0	46.5
H	7.7	7.0	7.6	6.0	6.7	7.1
O	23.9	28.6	26.9	34.5	32.7	28.3
N	6.0	7.2	5.9	8.6	8.3	7.5
S	0.5	0.5	0.4	0.7	0.7	0.7
Ash	4.9	6.1	3.8	9.2	7.7	8.0
Total Dry matter, mg.	26	22	26	12	17	21
Water, mg.	1	142	55	203	303	209
Total Plant Wt., mg.	27	164	81	215	320	230

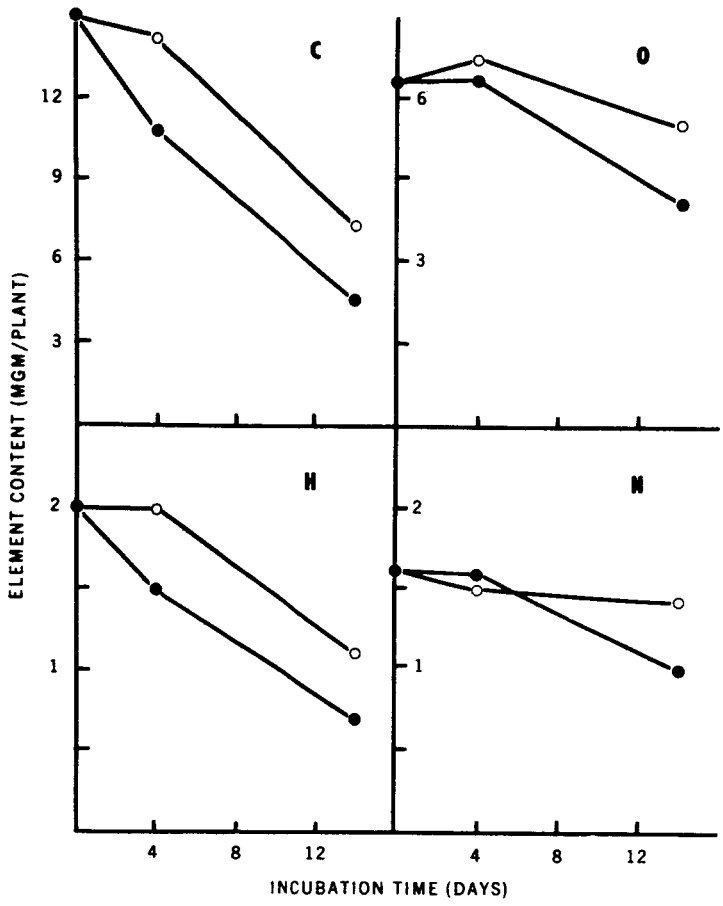


Fig. III-C1 - Changes in the C, H, O and N Content of Dark-Grown Cucumber Seedlings in Air (● - ●) or 5% O_2 + 95% Argon (o - o).

small loss in H was noted in the low O_2 group, whereas a gain in O relative to the seed occurred.

During the first four days of incubation, relatively little C was lost, O increased slightly, and no detectable loss in H occurred. Although no quantitative measurements of gaseous products were made at that time, the vessel contained CO_2 and CO as well as water vapor, O_2, and argon.

The low-oxygen seedlings at four days averaged 5.9 mm in hypocotyl length and 26.9 mm in root length in spite of their failure to expend appreciable tissue hydrogen via an aerobic oxidative system. The complete oxidation of carbohydrate (as glucose) would entail a loss of 2 atoms of H per atom of C. Oxidation of lipid (as linoleic acid) would require about 1.6 atom per atom of C. In the present case, however, approximately 0.4 atoms H were lost per atom C at 4 days, a figure which remains to be explained.

The initial increase in tissue O noted suggests short-term oxidations involving direct addition of molecular oxygen rather than dehydrogenation.

Carbon monoxide was first detected in the atmosphere of cucumber seedlings which had been grown from seeds at 25°C in 5% oxygen + 95% argon. The atmosphere in sealed growth jars as previously described was adjusted to 5.0% O_2, 0.002% CO_2 after 4 days' incubation, and the jars were placed in darkness for 7 days.

At the end of this period, the O_2 content was unchanged, and CO_2 had risen to about 3.5%, and contained approximately 6000 ppm of CO. The jars (gas volume, 7000 cm^3) contained 38 completely achlorophyllous seedlings totaling 12 g. in fresh weight. Accordingly, some 4.2 mg of CO per gram of fresh weight had been generated. It should also be noted that the 38 seedlings doubled in height during this period, elongating some 30 mm on the average.

In a subsequent experiment, cucumber seeds were germinated in an atmosphere containing $< 0.5\%$ O_2 and about 0.24% CO_2 in argon. After 10 days in darkness, when the seedlings had grown to 15 mm in height, their atmosphere showed no change in O_2, increased CO_2 (1.5%), and 0.04% (400 ppm) CO. During an additional 8 days

in darkness, the seedlings increased approximately 20 mm more in height. At that time, oxygen in the atmosphere continued unchanged, but CO_2 had increased to more than 5%, and CO had fallen to 10 ppm.

Cucumber seedlings grown in air produce no detectable CO, whether maintained in air or placed for as long as 7 days in low oxygen.

Preliminary analyses for DNA, RNA and protein have been run on cucumber seedlings grown for 2 weeks in 10% O_2 + 90% N_2. RNA was determined by the orcinol + $FeCl_3$ method; DNA with diphenylamine; and protein with $CuSO_4$ + phenol reagent. Expressed as ratios the analyses yielded:

	$\frac{RNA}{DNA}$	$\frac{Protein}{RNA}$	$\frac{Protein}{DNA}$
Air	2.02	0.56	1.13
10% O_2 + 90% N_2	0.86	2.47	2.13

Without additional data, it can only be said that the changes in these ratios imply that significant alterations in the transfer of information in protein biosynthesis must have taken place, and perhaps in the replication process as well. The present state of genetic knowledge leads to the expectation that a change in DNA-RNA-Protein relations should involve (a) changes in the levels of different specific enzymes, and (b) variations in the protein moieties of specific enzymes. Expectation (a) has been verified by change in amounts of two oxidizing enzymes, ascorbic acid oxidase and peroxidase (Table III-C2). Ascorbic acid oxidase rises slightly whereas peroxidase falls markedly, suggesting that enzyme changes do not reflect a change in total but, rather, in specific, proteins.

Expectation (b) has been verified by demonstrating, via starch gel electrophoresis, that cucumber peroxidase is heterogeneous both in electrophoretic mobility and substrate range (Fig. III-C2). Changes with O_2 level therefore do indeed affect specific protein synthesis.

Table III-C2

Effect of O_2 Level on the Activity of Two Oxidases in Cucumber Seedlings

a. Ascorbic Acid Oxidase (units/unit fr. wt.)

	Air	5% O_2 + 95% Ar	$\frac{5\%\ O_2}{\text{Air}}$
Leaf	8.86	9.12	1.03
Stem	1.42	1.65	1.16

b. Peroxidase (units/unit fr. wt.)

Leaf	6.76	3.17	0.47
Stem	1.32	0.83	0.63

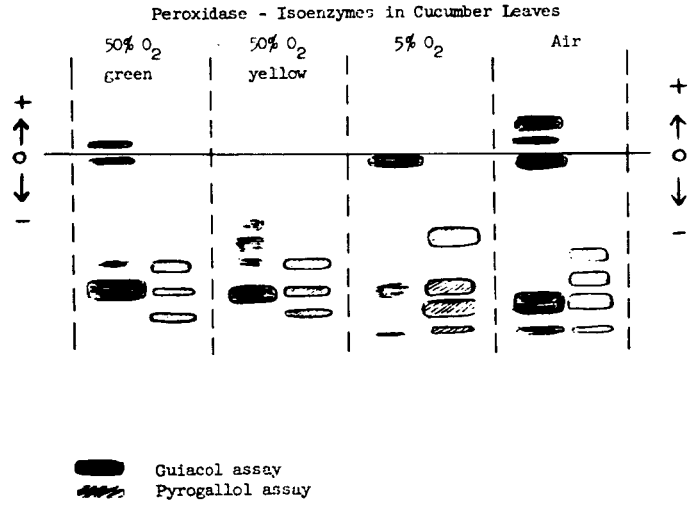


Fig. III-C2 - Iso-Peroxidases in Cucumber Seedlings Grown in Various O_2 Atmospheres. "Green" designates seedlings of normal healthy appearance in 50% O_2; "Yellow" designates seedlings exhibiting advanced senescence (20% of population) in 50% O_2.

From the preceding information, it is reasonable to propose the sequence:

Altered O_2 Level	⟶	Altered DNA-RNA-Protein Relationship	⟶	Changes in the Protein of Specific Enzymes	⟶	Altered Metabolism and Biosynthesis	⟶	Altered Growth and Form

But only the future will reveal its generality.

D. Pigments in Rye Seedlings - Rye seedlings can make chlorophyll when seeds are germinated in Ar, H_2, N_2, CO, and N_2O, as well as low partial pressures of O_2. It is obvious that there are many questions to be answered about biosynthetic processes in these unusual atmospheres.

Quantitatively, the appearance of chlorophylls a and b, and red anthocyanin pigment have been compared in rye grown in air and in 1% O_2 + 99% Ar. The emerging coleoptiles were harvested over several days. Growth, coloration, and chlorophyll from acetone extracts are recorded in Table III-II. With respect to growth and chlorophyll a, day 7 at 1% O_2 is similar to day 3 in air. This relationship does not apply to chlorophyll b, however, as it has risen on day 7 to nearly threefold more than the amount in 3-day air-grown plants. Thus, the ratio a/b which is in the ordinary range in air is changed markedly in low O_2. Furthermore, no red pigment whatsoever appeared up to day 7 in low oxygen plants.

E. Effects of Sub-freezing Temperatures on Plant Tissue Chemistry - Only the atmospheric factor has been examined to any degree with respect to biochemical changes. A few observations have been made, however, with xerophyte (succulent) leaves exposed to liquid nitrogen for 10 seconds, and with xerophytes kept under winter conditions in various atmospheres for 3 months.

Succulent leaves exposed to acute freezing in liquid N_2 show the following changes during several subsequent days at 25°C:

a. Slight decrease in Millon reagent-positive protein.

b. A more marked drop in Benedict-positive reducing sugar.

c. An increase in Schiff-positive aldehyde.

d. An enormous increase in KI_3-positive starch.

Table III-D1

A Comparison of Rye Coleoptile Pigments in Seedlings Grown in Air or 1% O_2 + 99% Ar

Incubation Time (Days)	Coleoptile, mm	Red Color*	Chlorophylls (μgms/gm)			
			a	b	a+b	a/b
in Air						
2	13	+	42	20	62	2.1
3	34	++	81	19	100	4.3
6	16	-	441	119	560	3.7
1% O_2						
3	15	-	9	2	11	-**
7	35	-	98	52	150	1.9

* Extracted from acidified ethanol with n-amyl alcohol. Typical anthocyanin λ_{max} at 540 mμ.

** Uncertainty in these low figures makes ratio meaningless.

e. Sizeable increases in the enzymes peroxidase and phosphorylase.

f. An enormous increase in Molybdate-positive ortho-diphenols.

Leaf pigmentation is grossly unchanged. These leaves are still viable; in soil, they can produce plantlets from the leaf base.

Chronic exposure (i.e., prolonged, but moderate stress) of succulent tissues to cold also brings about changes, but is strongly dependent upon O_2 level during the freezing period. In the presence of air, chlorophyll was rapidly lost and the leaf color became a bleached yellow. When only traces of O_2 were present, chlorophyll was normal in spectrum. Peroxidase, phosphorylase, aldehydes, reducing sugars, and starch were lacking in air-grown-plants. Air plants were dead, whereas those under N_2 were still viable, if not vigorous.

F. Changes in the Turtle at Reduced Pressure: Preliminary Notes - Turtles returned to 760 mm air after 54 days at 70-80 mm air appeared entirely normal in head and eye movements and limb coordination — swimming and walking were normal. The one external distinction noted was the formation observably over a period of hours of heavy, black circumorbital rings which appeared to be regular zones of melanization rather than typically irregular hematomas. After several weeks in air, these rings faded and slowly vanished.

Control and experimental animals were given preliminary hematological analyses (Table III-F1). Very little can be said at this time, except to note the following with increasing time at low pressure:

a. Red count is irregular, but may exhibit a falling trend.

b. White count fluctuates irregularly.

c. Hemaglobin content is falling markedly.

d. Specific white cell types are becoming less diversified.

Exploratory starch-gel electrophoresis of control and 34-day sera showed no γ-globulin in either sample; a strong control α_3-globulin had no counterpart in the low-pressure serum. In all, 12 bands were resolved in control turtle serum, although most are still not identified. In the low pressure group only 5 of these "normal" bands were still present, but one new band has actually appeared.

Table III-F1

Hematological Changes in the Blood of *Pseudemys* Maintained at P_{air} = 0.1 Atm.

a. General Analysis

Days at Reduced Pressure	Erythrocyte Count (per mm^3)	Leucocyte Count (per mm^3)	Hemoglobin gm/100 cc	Hematocrit (Vol. Formed Elements)
None (Controls)	347,250 ± 70,000	15,560 ± 6,000	4.88 ± 1.1	19.9 ± 0.8
19	462,000	16,070	2.25	17
34	90,000	12,900	2.60	16
54	220,000	18,500	1.25	--

b. Differential White Cell Analysis

Days at Reduced Pressure	Percentage of Monocytes	Juv. Metamyelocytes	Lymphocytes	Neutrophiles	Eosinophiles
None	92	2	3	1	2
34	76	1	15	5	3
54	99	0	0	0	1